Multitissue Transcriptomics Delineates the Diversity of Airway T Cell Functions in Asthma

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Abstract

Asthma arises from the complex interplay of inflammatory pathways in diverse cell types and tissues. We sought to undertake a comprehensive transcriptomic assessment of the epithelium and airway T cells that remain understudied in asthma and investigate interactions between multiple cells and tissues. Epithelial brushings and flow-sorted CD3⁺ T cells from sputum and BAL were obtained from healthy subjects ($n = 19$) and patients with asthma (mild, moderate, and severe asthma; $n = 46$). Gene expression was assessed using Affymetrix HT HG-U133⁺ PM GeneChips, and results were validated by real-time quantitative PCR. In the epithelium, IL-13 response genes (*POSTN*, *SERPINB2*, and *CLCA1*), mast cell mediators (*CPA3* and *TPSAB1*), inducible nitric oxide synthase, and cystatins (*CST1*, *CST2*, and *CST4*) were upregulated in mild asthma, but, except for cystatins, were suppressed by corticosteroids in moderate asthma. In severe asthma—with predominantly neutrophilic phenotype—several

distinct processes were upregulated, including neutrophilia (*TCN1* and *MMP9*), mucins, and oxidative stress responses. The majority of the disease signature was evident in sputum T cells in severe asthma, where 267 genes were differentially regulated compared with health, highlighting compartmentalization of inflammation. This signature included IL-17-inducible chemokines (*CXCL1*, *CXCL2*, *CXCL3*, *IL8*, and *CSF3*) and chemoattractants for neutrophils (*IL8*, *CCL3*, and *LGALS3*), T cells, and monocytes. A protein interaction network in severe asthma highlighted signatures of responses to bacterial infections across tissues (*CEACAM5*, *CD14*, and *TLR2*), including Toll-like receptor signaling. In conclusion, the activation of innate immune pathways in the airways suggests that activated T cells may be driving neutrophilic inflammation and steroid-insensitive IL-17 response in severe asthma.

Keywords: asthma; transcriptomics; epithelial brushings; sputum; BAL

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Clinical Relevance

Our data provide direct evidence that a global asthmatic signature is evident across tissues and that airway T cells are involved in orchestrating inflammation in severe asthma, characterized by activation of steroid-insensitive IL-17 responses and T cell signatures typical of responses to bacterial infections.

Asthma is a complex, chronic inflammatory disease of the airways, characterized by inflammation involving the interplay of a multitude of cell types, including mast cells, eosinophils, B cells, neutrophils, and airway smooth muscle cells, in interaction with the epithelium (1, 2). Airway T cells are believed to play a central role in driving these diverse pathobiological processes (3). The critical role of T-helper cells type 2 (Th2), producing type 2 cytokines, has long been appreciated, but there is an emerging recognition of a wide variety of novel T cell subsets and innate-like lymphocytes producing a diversity of cytokines, many of which may be steroid resistant (3, 4). Indeed, although the majority of patients with asthma respond to regular, low-dose inhaled corticosteroids (ICS), 5–10% of subjects with asthma suffer from severe disease, with persistent symptoms despite anti-inflammatory therapy, and often characterized by neutrophilic inflammation (5, 6).

Multiparameter flow cytometry has enabled detailed characterization of T cell subsets in the airways according to expression of surface makers and cytokine secretion (3). However, the range of markers that can be studied simultaneously is limited, and results will be restricted by the *a priori* selection of markers and hypotheses to be tested. Therefore, a powerful complementary approach is the application of unbiased, hypothesis-generating 'omics technologies. Several studies in asthma have used transcriptomics analysis to detect asthmatic gene signatures in different tissues, such as epithelium (7–9), whole sputum (10), and bronchial biopsies (11). However, both an analysis of the transcriptome of airway T cells and a multitissue investigation of the interaction between these different cells are lacking.

Our objective was to undertake a gene expression analysis of epithelial brushings and airway T cells from sputum and BAL to study the interactions between these tissues, across a range of human asthma. We therefore capitalized on both the unique capacity of flow cytometry to sort cells with high precision, and the power of an unbiased transcriptomic approach to synthesize a global view of the multiple, diverse pathobiological processes orchestrated by T cells in asthma. Results were validated in a subset of the patients by real-time quantitative PCR (qPCR).

Methods

Study Participants

65 participants (18–70 yr) were enrolled from the Wessex Severe Asthma Cohort, National Institute for Health Research Southampton Respiratory Biomedical Research Unit and outpatient clinics at University Hospital Southampton as previously described (3): 19 healthy non-atopic participants, 15 mild patients with asthma on β_2 -agonists alone, 17 subjects with moderate asthma on ICS and 14 subjects with severe asthma with persistent symptoms despite high-dose ICS ($n = 14$) and oral corticosteroids ($n = 4$) (Table 1), classified on enrolment applying criteria used previously (3) (*see the data supplement*). The study was approved by the Southampton and South West Hampshire Research Ethics Committee B. All participants provided informed consent.

Sample Collection

Lung samples were obtained by hypertonic saline sputum induction, bronchoscopy, BAL, and endobronchial epithelial brushing, as previously described (3) (*see the data supplement*). Live CD3⁺ T cells were isolated from BAL and sputum samples by flow cytometry using a nine-color FACSAria cell sorter (BD Biosciences; *see Figure E1 in the data supplement*).

RNA Isolation and Microarray Hybridization

RNA was extracted using the Absolutely RNA Nanoprep Kit and quality assessed using Bioanalyzer 2100 (Agilent Ltd., UK). RNA was reverse transcribed, amplified by *in vitro* transcription with the Ovation Pico WTA system V2 (NuGEN Technologies), and hybridized to Affymetrix HT HG-U133⁺ PM GeneChips (Affymetrix) by Janssen Research

and Development. Gene expression data are available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE89809.

Microarray Analysis

Raw microarray gene expression data were normalized using robust multiarray averaging (12) and subjected to quality control procedures, as previously described (9). Low expressed genes and genes with low standard deviation across samples were removed from further analysis (*see further details in the data supplement*). Differentially expressed genes were identified using *limma* (13) in R (version 3.2.2; The R Foundation) and subjected to functional enrichment analysis. A protein-protein interaction network was created using the STRING database (version 9.1) (14) and visualized in Cytoscape (version 2.8.3). Deconvolution analysis to extract cell type-specific profiles was performed using CTen (15) and quantification of relative levels of distinct cell types on a per sample basis for sputum severe asthma samples was performed using CIBERSORT and the LM22 gene signature database (16).

Real-Time qPCR Analysis

Real-time qPCR was performed in a subset of patients with asthma and healthy subjects to confirm microarray gene expression, as described in the data supplement.

Statistical Analyses

For microarray analysis, a linear mixed-modeling approach was used for differential gene expression taking into account pairing of samples, and age and sex differences between groups. Only genes with a false discovery rate (FDR) P less than 0.05 corrected for multiple testing using the Benjamini–Hochberg method (17) were considered significant. For Gene Ontology (GO) analysis, FDR-corrected hypergeometric P less than 0.05 was collapsed using REVIGO (Rudjer Boskovic Institute), where P less than 0.05 (represented as $-\log_{10} P$ value) was considered significant. For cell-type deconvolution analysis using CTen, an enrichment score greater than 2 was considered significant. The cell signatures obtained using the LM22 database in CIBERSORT were compared using a two-tailed Student's t test, and an FDR-corrected P value less than 0.05 was considered significant. The IL-13- and

Table 1. Demographic and Clinical Characteristics of the Participants

Variable	Healthy Control Subjects	Mild Asthma	Moderate Asthma	Severe Asthma
<i>n</i>	19	15	17	14
Demographics				
Sex, M/F	13/6	8/7	7/10	7/7
Age (median [range]), yr	28 (20–65)	26 (21–64)	33 (21–56)	57 (31–67)
Pulmonary function				
FEV ₁ % predicted	108 (105–112)	88 (86–102)	100 (90–107)	63 (52–75)
FEV ₁ reversibility, %	4.7 (2.3–7.9)	13 (11–18)	12 (8.9–17)	19 (10–26)
PEFR % predicted	102 (94–111)	98 (90–107)	96 (91–99)	69 (58–81)
PEFR variability, %	0 (0–11)	17 (11–20)	21 (17–29)	14 (11–23)
PD ₂₀ , mg methacholine	Negative	0.19 (0.061–0.59)	0.22 (0.097–0.53)	Not tested
Exhaled nitric oxide, ppb, at 50 L/s	19 (11–23)	52 (29–106)	27 (14–46)	22 (15–39)
Clinical				
Atopy, skin test positive, Y/N	0/19	15/0	15/2	9/5
No. of skin test allergens positive	0 (N/A)	6 (4–7)	3 (3–5)	4 (4–6)
Peripheral eosinophil count, 10 ⁹ /L	0.1 (0.1–0.2)	0.2 (0.1–0.4)	0.2 (0.2–0.3)	0.2 (0.1–0.2)
Total IgE, IU/ml	18 (8.0–43)	172 (48–365)	119 (56–188)	84 (19–552)
Body mass index, kg/m ²	25.8 (23.6–28.8)	23.6 (22.7–26.5)	25 (23.0–32.0)	33.6 (28.3–41.8)
Smoking status				
Never	16	14	13	9
Former, mean pack-years	3 (3.7)	1 (5)	4 (1.8)	4 (27)
Current, mean pack-years	0	0	0	1 (49)
Duration of asthma, yr	N/A	18 (15–25)	22 (11–24)	43 (15–50)
ACQ score	N/A	0.6 (0.50–1.1)	1.3 (0.71–1.7)	2.8 (2.4–3.4)
GINA level of control, <i>n</i> (%)				
Controlled	N/A	8 (53)	3 (18)	0 (0)
Partly controlled	N/A	6 (40)	11 (65)	0 (0)
Uncontrolled	N/A	1 (6.7)	3 (18)	14 (100)
Treatment				
Inhaled steroids	No	No	Yes	Yes
Dose, equivalent µg BDP	N/A	N/A	400 (400–800)	1440 (1,280–1,920)
Maintenance oral corticosteroids, Y/N	No	No	No	4/10
Mean dose if taken, mg prednisolone/d		0	0	8.1
Short acting β agonist, Y/N	No	Yes	Yes	Yes
Long acting β agonist, Y/N	No	No	7/10	14/0
Leukotriene receptor antagonist, Y/N	No	No	0/17	11/3
Step on GINA treatment algorithm	N/A	1	2–3	4–5
Inflammatory subtype, <i>n</i> (%)				
Neutrophilic	4 (33)	3 (23)	3 (21)	8 (67)
Eosinophilic	0 (0)	2 (15)	2 (14)	2 (17)
Mixed granulocytic	0 (0)	0 (0)	0 (0)	1 (8.3)
Paucigranulocytic	8 (66)	8 (62)	9 (64)	1 (8.3)
Sputum cell differential, %				
Macrophages	52 (31–63)	49 (36–62)	53 (31–63)	24 (16–34)
Neutrophils	34 (8.0–64)	34 (24–46)	24 (14–51)	71 (61–79)
Epithelial	4.9 (2.4–21)	4.3 (3.3–9.0)	6.5 (1.5–17)	1.3 (0.13–6.5)
Eosinophils	0.38 (0.0–0.75)	1.5 (0.75–1.8)	1 (0.25–1.3)	0.5 (0.0–2.5)
Lymphocytes	0.25 (0.0–0.81)	0.25 (0.0–0.75)	0 (0.0–0.50)	0 (0.0–0.22)
BAL cell differential, %				
Macrophages	82 (74–88)	70 (60–77)	81 (75–85)	72 (46–87)
Neutrophils	2.6 (1.9–6.0)	2.5 (1.8–4.5)	3.5 (1.8–5.9)	7.5 (5.0–22)
Epithelial	11 (4.7–17)	21 (15–35)	12 (7.3–18)	9.5 (3.5–11)
Eosinophils	0.5 (0.0–0.69)	2 (1.0–3.0)	1 (0.19–2.6)	0 (0.0–1.0)
Lymphocytes	1.4 (1.0–2.1)	1.5 (0.75–3.0)	1.5 (0.69–2.1)	0.75 (0.0–1.3)
Relevant comorbidities, <i>n</i> (%)				
Allergic rhinitis	0 (0)	12 (80)	8 (47)	5 (36)
Nasal polyps	0 (0)	0 (0)	1 (5.9)	4 (29)
Eczema	2 (11)	7 (47)	5 (29)	2 (14)
Hypertension	1 (5.2)	0 (0)	2 (12)	3 (21)
Gastroesophageal reflux	0 (0)	0 (0)	2 (12)	2 (14)
Other (<i>n</i> = 1 each)	Urticaria	Hypothyroidism	Addison's disease, bronchiectasis, multiple sclerosis, benign intracranial hypertension	Neurofibromatosis I, hypothyroidism, psoriasis

Definition of abbreviations: ACQ = asthma control questionnaire; BDP = beclomethasone dipropionate; GINA = Global Initiative for Asthma; N/A = not available; PD₂₀ = provocative dose causing a 20% fall in FEV₁; PEFR = peak expiratory flow rate.

Values are median (interquartile range), unless stated otherwise. The inflammatory subtype is based on sputum differentials using the following cut-points: neutrophilic, >61%; eosinophilic, >3%. Percentages given are derived from those subjects with valid data.

IL-17–inducible chemokine signatures were compared by one-way ANOVA in Prism 6.0 (GraphPad Software, Inc.), with *P* less than 0.05 considered significant.

Results

Evaluation of Gene Expression Signature across Compartments and the Disease Spectrum

The overall gene expression profile of all samples was evaluated using principal component analysis. CD3⁺ T cells displayed a distinct profile from epithelial brushings, but not between BAL and sputum sources, primarily reflecting cell type differences (see Figure E2). Moreover, the smoking status of the subjects did not have a significant effect on the overall gene expression profile, with former smokers and current smokers clustering with the nonsmokers (see Figure E3). Smokers were thus retained for further analysis. Differentially expressed genes were identified between subjects with mild, moderate, and severe asthma, in turn, and healthy control subjects within each tissue site (Figure 1, Table 2, Tables E2–E7). The majority of the differential gene expression signal was evident in patients with severe asthma, primarily in CD3⁺ T cells isolated from sputum (Table 2), highlighting the inadequacy of current corticosteroid therapy in reversing disease-related gene expression changes in sputum.

Mild and Moderate Asthma

In the epithelium in mild asthma, IL-13 response genes (*POSTN*, *SERPINB2*, and *CLCA1*) and proteases that are products of mast cells (*CPA3* and *TPSAB1*), as well as inducible nitric oxide synthase (*NOS2*), were upregulated compared with health (Figure 1A, Table E2). These responses, which are corticosteroid responsive (7), were not significant in subjects with moderate asthma, suggesting that treatment is effective in clearing this disease signature in subjects with moderate ICS-treated asthma. Moreover, the stress/stimulus-response genes (*SCNNIG*, *LTF*, and *C4A*) were downregulated in the epithelium in subjects with moderate asthma (Figure 1B, Table E3). In contrast, cystatins (*CST1*, *CST2*, and *CST4*) were highly upregulated in both subjects with mild and moderate asthma compared with health. The differential expression of *SERPINB2*, *POSTN*, and *CPA3* in mild asthma and

CST1 in mild and moderate asthma was confirmed by real-time qPCR (Figure 2A).

In CD3⁺ T cells isolated from sputum, cellular processes, such as GO-annotated “cell cycle processes” were upregulated in both subjects with mild and those with moderate asthma compared with healthy control subjects (Figures 1D and 1E, Tables E5 and E6). As activation induces quiescent T cells to leave cell cycle arrest, this suggests there is increased activation of airway T cells in asthma. However, “immune response” was observed in subjects with moderate asthma only, showing the significant effect of disease in this group of patients in the sputum compartment. In CD3⁺ T cells isolated from BAL, there were no genes in subjects with mild asthma and only one gene (*SCGB1A1*) in subjects with moderate asthma that was differentially expressed compared with healthy control subjects, suggesting a lesser impact of disease on gene expression in this compartment. This is consistent with sputum being more representative of inflammation in the proximal airways, whereas BAL samples more distal compartments, which we have recently shown to have different transcriptomic profiles (9). These results indicate the significant impact of asthma in inducing disease-related gene expression changes in the epithelium and CD3⁺ T cells isolated from sputum.

Severe Asthma

The large numbers of differentially expressed genes identified when comparing severe asthma to healthy control subjects (Table 2, Figure 1) were combined across compartments (epithelium, sputum, and BAL; *n* = 293) to understand the interaction of genes between these cell types and their contribution to disease. GO analysis was performed with terms being assigned arbitrary colors (Figure 3A) “defense response” and “immune system process” were assigned the same color due to significant overlap in genes). Gene signatures typical of responses to bacterial infection were prominent as evidenced by GO analysis (Figure 3A) and by the upregulation of *CEACAM5* (18) in the epithelium and *CD14* and *TLR2* in sputum T cells (Table E4 and E7). *Haemophilus influenzae* was identified in the BAL sample from one of the seven subjects with severe neutrophilic asthma analyzed by metagenomics and standard respiratory

bacterial culture, detected by both techniques. This patient’s symptomatology responded well to targeted antibiotics. Next, we investigated these findings using cell-type enrichment analysis (Figures E4A and E4B, Table E8) and additional flow-cytometric studies to confirm that these molecules are indeed expressed on the surface of T cells, being highest on the innate-like mucosal-associated invariant T cell subset (Figure E4C).

A protein interaction network was created for the combined list of differentially expressed genes (shown as nodes), demonstrating known direct interactions (shown as edges) between the protein products of these genes in these tissues (Figure 3B). Prominent features of the network are recruitment of neutrophils, innate immune activation, and responses to bacterial products. Proteins involved in specific GO terms are highlighted in the network with respective colors. The “*Toll-like receptor signaling pathway*” (Kyoto Encyclopedia of Genes and Genomes ID, hsa04620) was also significantly enriched in sputum T cells, and the protein products of the genes involved in this pathway are highlighted in the protein interaction network. Differential expression of *CEACAM5*, *TCN1*, and *TLR2* was confirmed by real-time qPCR (Figure 2B). These results indicate the effect of severe asthma in epithelial brushings and T cells obtained from sputum in activating gene expression linked to innate immune pathways.

IL-13– and IL-17–Inducible Chemokines

The expression levels of the IL-13 (*POSTN*, *SERPINB2*, and *CLCA1*) and IL-17– (*CXCL1*, *CXCL2*, *CXCL3*, *IL8*, and *CSF3*) –inducible chemokines—established by Choy and colleagues (4)—were plotted for all tissues across all asthma severities relative to their respective healthy controls (Figure 4) to evaluate Th2 and Th17 signatures. The corticosteroid-responsive IL-13–inducible chemokines were highly expressed in epithelial samples obtained from subjects with mild asthma, but diminished with an increase in asthma severity (Figure 4A). Expression of these genes did not differ in sputum or BAL T cells. In contrast, the IL-17–inducible chemokines were only modulated in asthma samples obtained from sputum T cells, and their relative expression increased with an increase in asthma

Figure 1. Heat maps depicting unsupervised hierarchical clustering of samples and differentially expressed genes in (A) mild, (B) moderate, and (C) severe asthma in epithelium and (D) mild, (E) moderate, and (F) severe asthma in sputum, compared with healthy controls. Distances were calculated using Euclidean correlation metric and clustered using Ward's method. Gene expression values were averaged and scaled across the row to indicate the number of standard deviations above (red) or below (blue) the mean, denoted as row z-score. Color bars at the bottom represent healthy volunteers (black) and subjects with mild or moderate asthma (gray). *, #, §, ¥, Δ, Ψ, and Ω represent those samples in epithelium that did not cluster in their respective groups but had paired samples in sputum that clustered perfectly within the respective groups.

Table 2. Numbers of Differentially Expressed Genes

Sample Source	Cell Type	Differentially Expressed Genes (Compared to Healthy Controls)		
		Mild Asthma	Moderate Asthma	Severe Asthma
Epithelium	Mixed	14 (↑14; ↓0)	6 (↑3; ↓3)	25 (↑7; ↓18)
BAL	CD3 ⁺	0	1 (↑0; ↓1)	1 (↑0; ↓1)
Sputum	CD3 ⁺	14 (↑10; ↓4)	22 (↑16; ↓6)	267 (↑166; ↓101)

In the parenthesis next to each number, the number of upregulated genes is indicated, followed by the number of downregulated genes.

severity, but not in BAL T cells or epithelium (Figure 4B). An additional analysis was performed in subjects with paired samples from epithelium and sputum (21 subjects with asthma and 9 healthy control subjects) with similar results (Figure E5), suggesting that these differences were not a result of nonadherence to therapy. These results suggest that the corticosteroid therapy works effectively against the IL-13-inducible chemokines that are primarily modulated in epithelium,

whereas better therapies targeted toward the IL-17-inducible chemokines in sputum are needed for the management of severe asthma.

Discussion

By applying a transcriptomic analysis to investigate inflammatory processes in multiple tissues, across a spectrum of asthma, we have demonstrated a wide variety of inflammatory pathways in which

airway T cells are implicated. As visualized in the rich protein interaction network (Figure 3B), the striking number of differentially expressed genes in sputum T cells, and the perfect separation of health and asthma by hierarchical clustering of sputum T cell data (Figures 1D–1F), our findings highlight the diverse, central roles that T cells play in orchestrating the pathobiology of asthma.

Using untargeted global gene expression profiling, a robust IL-13 response signature (*CLCA1*, *POSTN*, and *SERPINB2*), as well as a mast cell signature, was evident in subjects with mild asthma, providing important validation of previous studies in mild disease (7, 8). There was also a prominent upregulation of cystatins (*CST1*, *CST2*, and *CST4*) in mild and moderate asthma, with *CST1* being the most upregulated gene in both mild and moderate disease, suggesting an important role for these molecules in asthma. Cystatins are small proteins present in all biological fluids, which are potent, reversible, competitive inhibitors of

Figure 2. Quantitative real-time qPCR validation of microarray findings in (A) subjects with mild and moderate asthma for genes *SERPINB2*, *POSTN*, *CPA3*, and *CST1* in epithelial brushings and in (B) subjects with severe asthma for genes *CEACAM5* and *TCN1* in epithelial brushings, and *CD14* and *TLR2* in sputum. Statistical significance was assessed by Mann–Whitney (two groups) and Kruskal–Wallis with *post hoc* Dunn’s (multiple groups) tests. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. ns = not significant.

Figure 3. Functional enrichment of combined differentially expressed gene list in severe asthma across all tissues. (A) Gene Ontology (GO) analysis with arbitrary colors assigned to biological GO terms. Defense response and immune system process were assigned the same color owing to a large overlap in genes in these terms. GO terms with false discovery rate (FDR)-corrected P less than 0.05 were collapsed into categories of related terms using REVIGO, where $-\log_{10} P$ values were considered significant. A red line indicating P less than 0.05 is shown here. (B) Protein interaction network representing the interactions of the protein products of differentially expressed genes. The size of the node is reflective of the number of interactions, and the color key indicates upregulation (red) or downregulation (blue) compared with healthy controls. Triangles, squares, and circles represent BAL, epithelium, and sputum, respectively. Functionally related genes are grouped into colors reflecting their respective GO terms from A. Toll-like receptor signaling Kyoto Encyclopedia of Genes and Genomes pathway (FDR-corrected $P < 0.05$) is indicated with a dashed line.

cysteine proteinases (cathepsins) (19) and, therefore, have recognized roles in inflammation. In addition cystatins have unrelated potent immunomodulatory functions and can, for instance, induce synthesis of TNF- α and IL-10 (20). Recent studies have shown differential expression of *CST1* in airway epithelial cells in exercise-induced bronchoconstriction, and identified a SNP that controls expression of *CST1* and is associated with FEV₁ decline in asthma (21). Others have reported elevation of, cystatin C (*CST3*) in asthma

(22)—which we did not observe—and, more recently, of *CST2* (11). These steroid-sensitive IL-13 and mast cell responses and nitric oxide synthase were not prevalent in subjects with moderate asthma, and were abrogated in severe asthma (Figure 4A), likely due to high-dose therapeutic corticosteroids. Conversely, all three cystatin genes remained highly upregulated in moderate—though not in severe—disease (Tables E2–E4). Cystatins, therefore, warrant further investigation as potential novel therapeutic targets.

In contrast to the steroid-sensitive processes dominant in mild and moderate asthma, the IL-17-inducible chemokines (*CXCL1*, *CXCL2*, *CXCL3*, *IL8*, and *CSF3*) showed the highest expression in severe asthma (Figure 4B), consistent with this pathway being resistant to, or even enhanced by (4), corticosteroids. Interestingly, although Choy and colleagues (4) initially described this specific gene signature in cultured normal human bronchial epithelial cells treated with IL-17A and TNF- α , we did not observe this

Figure 4. Relative gene expression of IL-13- and IL-17-inducible chemokines. The microarray expression levels of the (A) IL-13- (*POSTN*, *SERPINB2*, and *CLCA1*) and (B) IL-17 (*CXCL1*, *CXCL2*, *CXCL3*, *IL8*, and *CSF3*)–inducible chemokines are plotted for all tissues across all asthma severities relative to their respective healthy controls. Black triangles, blue squares, and red circles represent BAL, epithelium, and sputum, respectively. Statistical significance was assessed by one-way ANOVA, with the color of asterisks and bars representing the significance in the respective tissues. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

signature in epithelial cells *in vivo*, but rather in sputum T cells. It is interesting that this complex of chemokines is not suppressed by steroids, whereas IL-17A itself is suppressed by dexamethasone (4). As neither IL-17A, nor flow-cytometrically measured airway Th17 cells were elevated in severe asthma in this patient cohort (3), this suggests that other cell types and processes—such as TNF- α secretion—may be responsible for the induction of these chemokines *in vivo*.

The protein interaction network (Figure 3B) explores the interaction of T cells and epithelium in severe asthma, illustrating the involvement of airway T cells in several key pathobiological processes. For instance, the recruitment of neutrophils in severe asthma may be driven by T cell–derived IL-8, which is prominent within the network, visible from the color intensity (upregulation) and size (connectivity) of this node. This node interacts closely with other molecules involved with granulocyte recruitment, including *CCL3* (C-C motif chemokine ligand 3/macrophage inflammatory protein 1- α), *LGALS3* (galectin-3, a regulator of neutrophil and eosinophil recruitment) (23), and *MMP9* (matrix metalloproteinase 9, a type IV collagenase involved with IL-8–induced mobilization of neutrophils, which we have shown is associated with neutrophilic asthma [6]). The particular strength of these signals in our dataset may

be related to the specific focus on neutrophilic asthma in this cohort (67% of the severe group).

One important T cell–epithelial interaction is between T cell sputum IL8, *LGALS3*, and the epithelial upregulation of *MUC5AC* (Figure 3B). Woodruff and colleagues (8) have previously described an increase in the ratio of epithelial *MUC5AC*: *MUC5B* expression in mild–moderate Th2-high asthma. We too observed repression of epithelial *MUC5B* in mild asthma, but also saw that *MUC5AC* was strongly upregulated in severe asthma, despite high-dose corticosteroids and predominantly neutrophilic inflammation, suggesting that this mechanism may also be common to steroid-resistant neutrophilic asthma.

A second important set of interactions in the protein interaction network (Figure 3B) involves upregulation of epithelial *SNCA* and *ALAS2* with activation of T cell pathways typical of responses to bacterial products, including *SLC11A1* (natural resistance-associated macrophage protein 1 [*NRAMP1*]), *RELA*, *JUN*, and *RIPK2*. Both these epithelial genes are implicated in response to oxidative stress and tobacco smoking. In a recent study of gene–environment interactions, SNPs in *SNCA* (α -synuclein) were identified as the most strongly associated with accelerated lung function decline induced by tobacco or air pollution (24). Likewise, *ALAS2*

expression mediates the association between smoking and production of IL-6 and C-reactive protein (25). In our dataset, we did not observe a dramatic effect of smoking on overall gene expression profile. This is likely because only one subject was a current smoker, so differences in expression profiles between tissues and related to asthma will have dominated the principle component analyses. However, although only one participant reported current smoking status, four other participants with severe disease had a significant previous smoking history; thus, differential expression of these genes could be related to current unreported smoking, passive exposure, or to persistent activation of processes initiated by previous tobacco exposure. However, these processes could equally be attributed to other sources of oxidative stress in asthma, particularly inflammatory cell–derived reactive oxygen species.

A third prominent feature of the protein interaction network (Figure 3B) is the upregulation in sputum T cells of pathways associated with innate immune activation, particularly *TLR2*, *CD14*, and *SLC11A1* (*NRAMP1*). The latter is expressed by innate lymphocytes and augments their activation, particularly in the $\gamma\delta$ T cell subset (26), biasing toward a strong IFN- γ response, and Th1 rather than Th2 responses (26). Expression of *TLR2* and *CD14* is classically associated

with innate immune cells, particularly monocytes (27, 28). However, we confirmed *CD14* protein expression by surface staining on T cells, finding the highest expression on the innate-like mucosal-associated invariant T cell subset (Figure E4C), a cell type that we have recently shown to play an important role in host defense against airways infection by bacteria, such as *H. influenzae* (29).

Furthermore, increased expression of *TLR2* and *CD14* has been reported on $CD8^+$ T cells in chronic obstructive pulmonary disease (30, 31), and in whole sputum in noneosinophilic asthma, associated with increased sputum endotoxin (32). Thus, upregulation in severe neutrophilic asthma of such innate pathogen receptors on T cells, together with other components of the NF- κ B signaling pathway and epithelial upregulation of *CEACAM5* (a receptor exploited by airway pathogens, including *H. influenzae* [18]), constitutes a signature suggestive of bacterial infection driving the inflammation of the airways in these individuals.

Finally, it should be noted that our protein interaction network recapitulates many other more well-described features of asthma, including high upregulation of *FCER1G* (the high-affinity IgE receptor), *PTGS2* (cyclo-oxygenase-2), *NOS2* (inducible nitric oxide synthase, the source of increased exhaled nitric oxide in untreated asthma) (1), and *CCL17* (chemotactic to $CCR4^+$ pulmonary T cells) (33), as well as downregulation of *IFNA21* (IFN- α), consistent with the known deficiency in type I IFN production (34). These lend confidence in the validity of the gene expression signals emerging from our

dataset and to the potential value in exploring other genes of interest that we have identified here. These include *CNTF* (ciliary neurotrophic factor), which has been implicated both in enhancing IgE production in allergic disease (35) and *in vitro* in a response to gastroesophageal reflux (36), an important comorbidity in severe asthma.

Our study has some limitations, and the results should be interpreted carefully. First, epithelial brushings are of mixed cell type, and investigations in pure, isolated cell populations would be ideal. Cell type enrichment tools could provide additional information, but these tools are still under development, and depend on specific marker genes, which are lacking for the cell subsets that comprise epithelial brushings. Moreover, the CIBERSORT analysis must be interpreted with care, as it is only an *in silico* analysis to identify relative levels of transcripts in complex gene expression data, and these gene signatures may, in fact, be present to different extents in different cell types. Nonetheless, others have demonstrated that airway epithelial cells comprise 95–97% of cells obtained by bronchial brushings in patients with airways disease, with proportions of airway macrophages and lymphocytes constant between health and severe disease (37). Second, our sample size is limited, and it will be interesting to replicate these findings in a future clinical cohort including a greater representation of severe eosinophilic phenotypes. In addition, there was an imbalance in atopy between cases and control subjects. A cohort of control

subjects with atopy in a future study would help differentiate gene expression changes related to asthma, atopy, or both. Third, we would like to have included data on T cells from collagenase-dispersed bronchial biopsies as perhaps the most clinically relevant tissue site. This was attempted, but cell numbers were limiting, providing insufficient RNA for analysis.

In summary, by examining transcriptomic signatures across multiple tissues and cell types, we have observed airway T cells orchestrating recruitment of neutrophils, monocytes, and T cells in severe asthma. We have generated important confirmatory evidence of the roles of IL-13- and IL-17-inducible chemokines and direct evidence of dysregulation *in vivo* of genes recently linked to genetic susceptibility to asthma and lung function decline. The innate immune signatures observed in both epithelium and T cells putatively suggest a pathogenic role for bacterial airways infection. Thus, our results support future investigation of therapies targeting IL-17-inducible chemokines and bacterial airways colonization in severe neutrophilic asthma. ■

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