

1 **Title:** Multi-tissue transcriptomics delineates the diversity of airway T cell
2 functions in asthma

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List of abbreviations

BAL: Bronchoalveolar lavage; Benjamini-Hochberg (BH); FACS:
Fluorescence activated cell sorting; FDR: False discovery rate; GO: Gene
Ontology; ICS: Inhaled corticosteroids; KEGG: Kyoto Encyclopedia of Genes
and Genomes; MAIT: Mucosal associated invariant T; RT-qPCR: Real-time
quantitative PCR

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Authors' contributions

AS performed the microarray data analysis, carried out the RT-qPCR
experiment and analysis and was involved in the study design and drafting the
manuscript. JCW performed the experiments to characterize the surface

50 expression of CD14 on subsets of CD3+ cells and appraised the manuscript.
51 CGS provided nursing support for recruitment and sample collection. DH
52 performed microarray hybridization. KJS supervised the CD3+ cell subset
53 experiment and edited the manuscript. PHH, SDG and RD contributed to
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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 bronchoalveolar lavage were obtained from healthy subjects (N=19) and asthmatic patients (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*, *CLCA1*), mast cell mediators (*CPA3*, *TPSAB1*), inducible nitric oxide synthase and cystatins (*CST1*, *CST2*, *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*, *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 to health, highlighting compartmentalisation of inflammation. This signature included IL-17-inducible chemokines (*CXCL1*, *CXCL2*, *CXCL3*, *IL8*, *CSF3*) and chemoattractants for neutrophils (*IL8*, *CCL3*, *LGALS3*), T cells and monocytes. A protein interaction network in severe asthma highlighted signatures of responses to bacterial infections across tissues (*CEACAM5*, *CD14*, *TLR2*) including toll-like receptor signalling. In conclusion, the activation of innate immune pathways in the airways suggests that activated T

85 cells may be driving neutrophilic inflammation and steroid-insensitive IL-17
86 response in severe asthma.

87 INTRODUCTION

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

101

102 Multi-parameter flow-cytometry has enabled detailed characterization of T cell
103 subsets in the airways according to expression of surface makers and
104 cytokine secretion (3). However, the range of markers that can be studied
105 simultaneously is limited, and results will be restricted by the *a priori* selection
106 of markers and hypotheses to be tested. Therefore a powerful complementary
107 approach is the application of unbiased, hypothesis- generating 'omics
108 technologies. Several studies in asthma have utilized transcriptomics analysis
109 to detect asthmatic gene signatures in different tissues such as epithelium (7-
110 9), whole sputum (10) and bronchial biopsies (11). However, both an analysis

of the transcriptome of airway T cells, and a multi-tissue 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 bronchoalveolar lavage, 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 synthesise 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.

MATERIALS AND METHODS

Study participants

65 participants (18-70 years) were enrolled from the Wessex Severe Asthma Cohort, NIHR Southampton Respiratory Biomedical Research Unit and outpatient clinics at University Hospital Southampton as previously described (3): 19 healthy non-atopic participants, 15 mild asthma patients on β 2-agonists alone, 17 moderate asthmatics on inhaled corticosteroids (ICS) and 14 severe asthmatics 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 online supplement). The study was approved by the Southampton and South West Hampshire Research Ethics Committee B. All participants provided informed consent.

136

137 **Sample collection**

138 Lung samples were obtained by hypertonic-saline sputum induction,
139 bronchoscopy, bronchoalveolar lavage (BAL) and endobronchial epithelial
140 brushing as previously described (3) (see online supplement). Live CD3+ T
141 cells were isolated from BAL and sputum samples by flow-cytometry using a
142 nine-colour FACS AriaTM cell sorter (BD Biosciences)(see online **Figure E1**).

143

144 **RNA isolation and microarray hybridization**

145 RNA was extracted using the Absolutely RNA Nanoprep Kit and quality
146 assessed using Bioanalyzer 2100 (Agilent Ltd., UK). RNA was reverse
147 transcribed, amplified by *in vitro* transcription with the Ovation Pico WTA
148 system V2 (NuGEN Technologies, San Carlos, USA) and hybridized to
149 Affymetrix HT HG-U133+ PM GeneChips (Affymetrix, California, USA) by
150 Janssen Research & Development (Pennsylvania). Gene expression data are
151 available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>)
152 under accession number GSE89809.

153

154 **Microarray analysis**

155 Raw microarray gene expression data was normalized using RMA (12) and
156 subjected to quality control procedures as previously described (9). Low
157 expressed genes and genes with low standard deviation across samples were
158 removed from further analysis (further details in online supplement).
159 Differentially expressed genes were identified using *limma* (13) in R (version
160 3.2.2) 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 carried out using CIBERSORT and the LM22 gene signature database (16).

RT-qPCR analysis

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

Statistical analyses

For microarray analysis, a linear mixed modelling approach was used for differential gene expression taking into account pairing of samples, and age and gender differences between groups. Only genes with false discovery rate (FDR) $p < 0.05$ corrected for multiple testing using the Benjamini-Hochberg (BH) method (17) were considered significant. For GO analysis, FDR corrected hypergeometric $p < 0.05$ were collapsed using REVIGO, where $p < 0.05$ (represented as $-\text{Log}_{10}$ p-value) was considered significant. For cell-type deconvolution analysis using CTen, an enrichment score > 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 $p\text{-value} < 0.05$ was considered significant. The IL-13 and IL-17

inducible chemokine signatures were compared by one-way ANOVA in Prism 6.0, with $p < 0.05$ 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 online **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 non-smokers (see online **Figure E3**). Smokers were thus retained for further analysis. Differentially expressed genes were identified between mild, moderate and severe asthmatics, in turn, and healthy controls within each tissue site (**Figure 1, Table 2**, see online **Tables E2 through E7**). The majority of the differential gene expression signal was evident in severe asthma patients, 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*, *TPSAB1*),

as well as inducible nitric oxide synthase (*NOS2*) were upregulated compared with health (**Figure 1A**, online **Table E2**). These responses, which are corticosteroid responsive (7), were not significant in moderate asthmatics suggesting that treatment is effective in clearing this disease signature in moderate ICS-treated asthmatics. Moreover, the stress/stimulus-response genes (*SCNN1G*, *LTF* and *C4A*) were downregulated in the epithelium in moderate asthmatics (**Figure 1B**, online **Table E3**). In contrast, cystatins (*CST1*, *CST2* and *CST4*) were highly upregulated in both mild and moderate asthmatics compared with health. The differential expression of *SERPINB2*, *POSTN* and *CPA3* in mild asthma and *CST1* in mild and moderate asthma was confirmed by RT-qPCR (**Figure 2A**).

In CD3⁺ T cells isolated from sputum, cellular processes such as GO-annotated '*cell cycle processes*' were upregulated in both mild and moderate asthmatics compared with healthy controls (**Figure 1D** and **1E**, online **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 moderate asthmatics 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 mild asthmatics and only one gene (*SCGB1A1*) in moderate asthmatics that was differentially expressed compared to healthy controls suggesting a lesser impact of disease on gene expression in this compartment, and is consistent with sputum being more representative of inflammation in the proximal airways whilst 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 controls (**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 colours (**Figure 3A**) ('*Defense response*' and '*immune system process*' were assigned the same colour 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 (online **Table E4** and **E7**). *Haemophilus influenzae* was identified in the BAL sample from one of the seven severe neutrophilic asthmatic subjects analysed 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 (see online supplementary data, **Figures E4A** and **E4B, Table E8**) and additional flow-cytometric studies to confirm these molecules are indeed expressed on the surface of T cells, being highest on the innate-like mucosal associated invariant T (MAIT) cell subset (online **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 colours. The '*toll-like receptor signalling pathway*' (KEGG 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 RT-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 *et al.* (4) – were plotted for all tissues across all asthma severities relative to their respective healthy controls (**Figure 4**) in order to evaluate Th2 and Th17 signatures. The corticosteroid responsive IL-13 inducible chemokines were highly expressed in epithelial samples obtained from mild asthmatics 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 severity, but not in BAL T cells or epithelium (**Figure 4B**). An additional analysis was carried out in subjects with paired samples from epithelium and sputum (21 asthmatic and 9 healthy controls) with similar results (online **Figure E5**), suggesting that these differences were not a result of non-adherence 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 towards 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 visualised 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, 1E and 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*, *SERPINB2*) as well as a mast cell signature was evident in mild asthmatics, providing important validation of previous studies in mild disease (7, 8). There was also a prominent upregulation of cystatins

(*CST1*, *CST2*, *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 cysteine proteinases (cathepsins) (19) and therefore have recognised 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 single nucleotide polymorphism which 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 moderate asthmatics, 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 severe – disease (online **Tables E2, E3 and 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 (4) by corticosteroids. Interestingly, whilst Choy *et al.* (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 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, whilst 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 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 colour 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 *et al.* (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 *MUC5AC* was strongly upregulated in severe asthma, despite high dose corticosteroids and predominantly neutrophilic inflammation, suggesting 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, single nucleotide polymorphisms 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 1 subject was a current smoker so differences in expression profiles between tissues and related to asthma will have dominated the principle component analyses. However, whilst only one participant reported current smoking status, four other severe participants 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 towards a strong IFN- γ response and Th1 rather than Th2 responses (26). Expression of *TLR2* and *CD14* are 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 MAIT cell subset (online **Figure E4C**), a cell type we have recently shown to play an important role in host defense against airways infection by bacteria such as *Haemophilus influenzae* (29). Furthermore increased expression of *TLR2* and *CD14* has been reported on CD8⁺ T cells in COPD (30, 31), and in whole sputum in non-eosinophilic 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 signalling pathway and epithelial upregulation of *CEACAM5* (a receptor exploited by airway pathogens including *Haemophilus 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* (cyclooxygenase-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* (interferon- α), consistent with the known deficiency in type I interferon production (34). These lends confidence in the validity of the gene expression signals emerging from our dataset and to the potential value in exploring other genes of interest 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 gastro oesophageal reflux (36); an important comorbidity in severe asthma.

Our study has some limitations and the results should be interpreted carefully. Firstly, 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 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). Secondly 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. Additionally, there was an imbalance in atopy between cases and controls. A cohort of atopic controls in a future study would help differentiate gene expression changes related to asthma, atopy, or both. Thirdly 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 colonisation in severe neutrophilic asthma.

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612 **TABLES**613 **Table 1. Demographic and clinical characteristics of the**614 **participants**

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Variable	Healthy Controls	Mild Asthma	Moderate Asthma	Severe Asthma
n	19	15	17	14
Demographics				
Sex (M/F)	13 / 6	8 / 7	7 / 10	7 / 7
Age (median [range], years)	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)
PD20 (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 (years)	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 (n, %)				
Controlled	N/A	8 (53)	3 (18)	0 (0)
Partly controlled	N/A	6 (40)	11 (65)	0 (0)

Uncontrolled Treatment	N/A	1 (6.7)	3 (18)	14 (100)
Inhaled steroids	No	No	Yes	Yes
Dose (equivalent mcg BDP)	N/A	N/A	400 (400-800)	1440 (1280-1920)
Maintenance oral corticosteroids (Y/N)	No	No	No	4 / 10
Mean dose if taken (mg prednisolone/day)		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 (n, %)				
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 (n, %)				
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)
Gastro-oesophageal reflux	0 (0)	0 (0)	2 (12)	2 (14)

Other (n=1 each)

Urticaria

Hypothyroidism

Addison's
disease,
bronchiectasis,
multiple sclerosis,
benign
intracranial
hypertension

Neurofibromatosis
I, hypothyroidism,
psoriasis

Values are medians with interquartile ranges, unless stated otherwise. N/A: not available. 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. Abbreviations are as follows: *ACQ*, asthma control questionnaire; *BAL*, bronchoalveolar lavage; *BDP*, beclometasone dipropionate; *CT*, computed tomograph; *FEV₁*, pre-bronchodilator forced expiratory volume in 1 second; *FVC*, forced vital capacity; *GINA*, Global Initiative for Asthma; *mcg*, micrograms; *mg*, milligrams; *PEFR*, peak expiratory flow rate; *PD₂₀*, provocative dose causing a 20% fall in *FEV₁*.

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Table 2. Numbers of differentially expressed genes

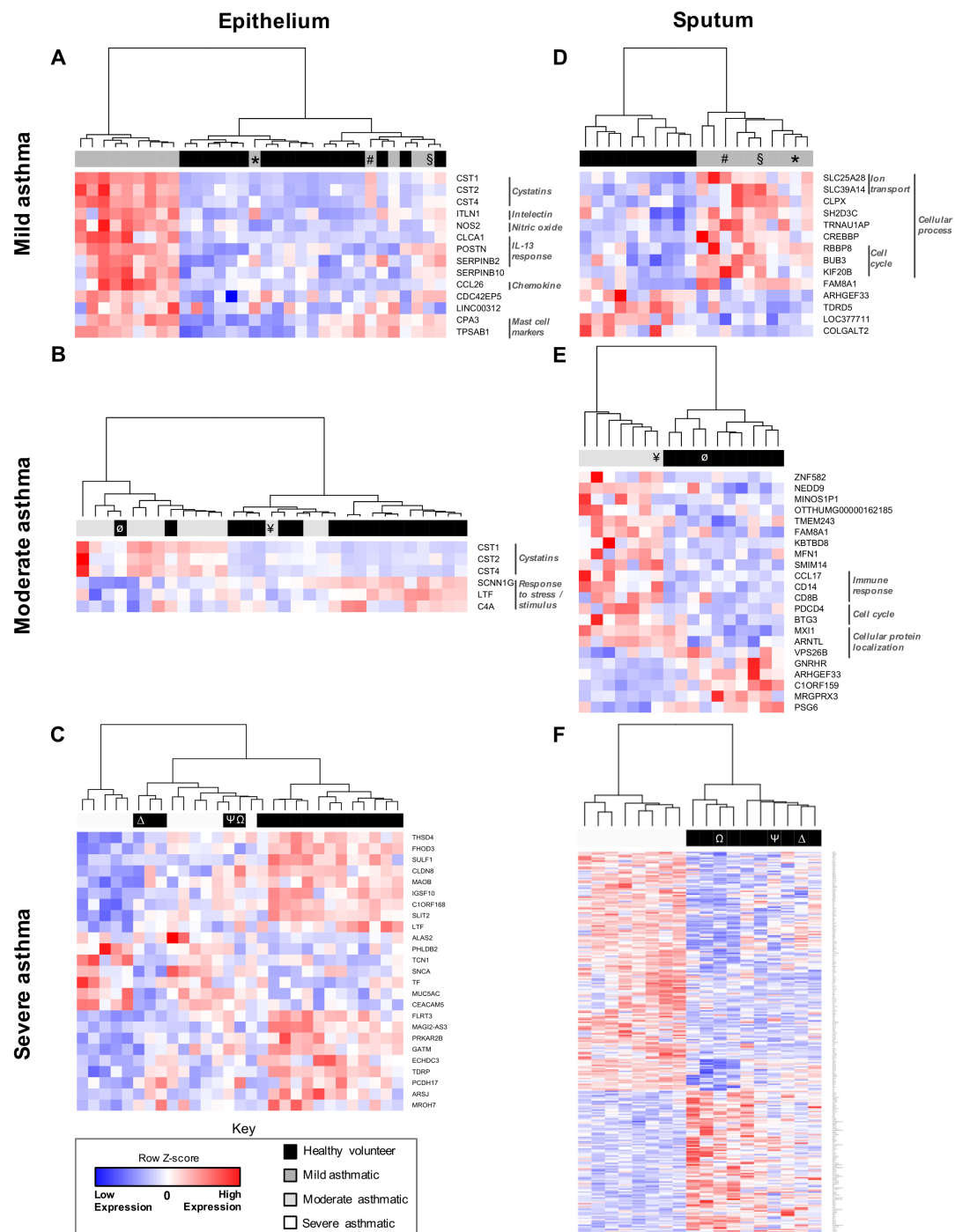
Sample source	Cell type	Differentially expressed genes (compared to healthy controls)		
		Mild Asthma	Moderate Asthma	Severe Asthma
Epithelium	Mixed	14 (↑14; ↓0)	6 (↑3; ↓3)	25 (↑7; ↓18)
BAL	CD3+	0	1 (↑0; ↓1)	1 (↑0; ↓1)
Sputum	CD3+	14 (↑10; ↓4)	22 (↑16; ↓6)	267 (↑166; ↓101)

BAL, bronchoalveolar lavage

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

FIGURES

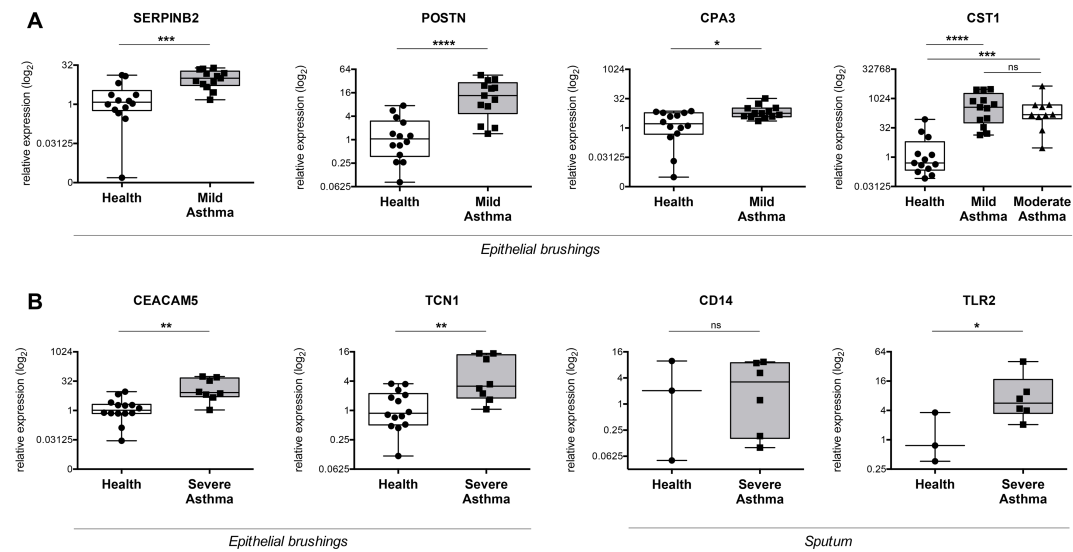
Figure 1.



Heatmaps 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 to healthy controls. Distances were calculated using

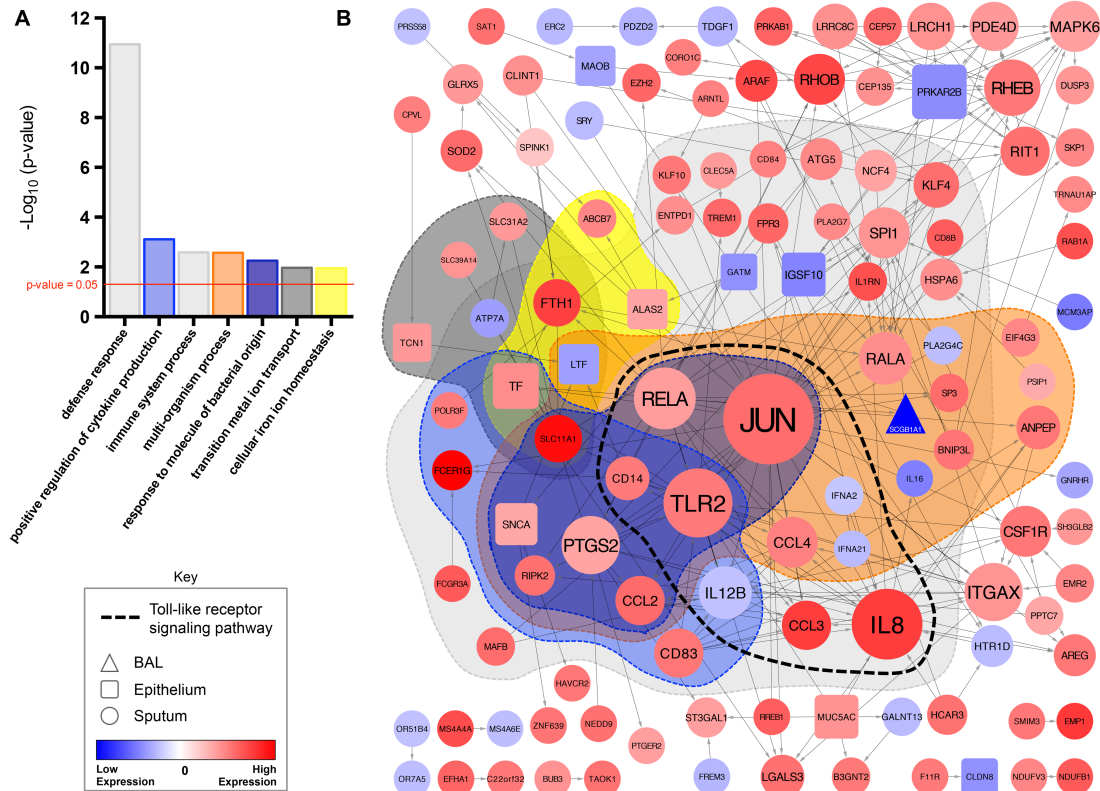
660 Euclidean correlation metric and clustered using Ward's method. Gene
661 expression values were averaged and scaled across the row to indicate the
662 number of standard deviations above (red) or below (blue) the mean, denoted
663 as row Z-score. Colour bars at the top represent healthy volunteers (black)
664 and mild or moderate asthmatics (grey). *, #, §, ø, ¥, Δ, Ψ and Ω represent
665 those samples in epithelium that did not cluster in their respective groups, but
666 had paired samples in sputum that clustered perfectly within the respective
667 groups.
668

Figure 2.



RT-qPCR validation of microarray findings in (A) mild and moderate asthmatics for genes *SERPINB2*, *POSTN*, *CPA3* and *CST1* in epithelial brushings and in (B) severe asthmatics 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. *, $P \leq .05$; **, $P \leq .01$; ***, $P \leq .001$; ****, $P \leq .0001$, ns, not significant.

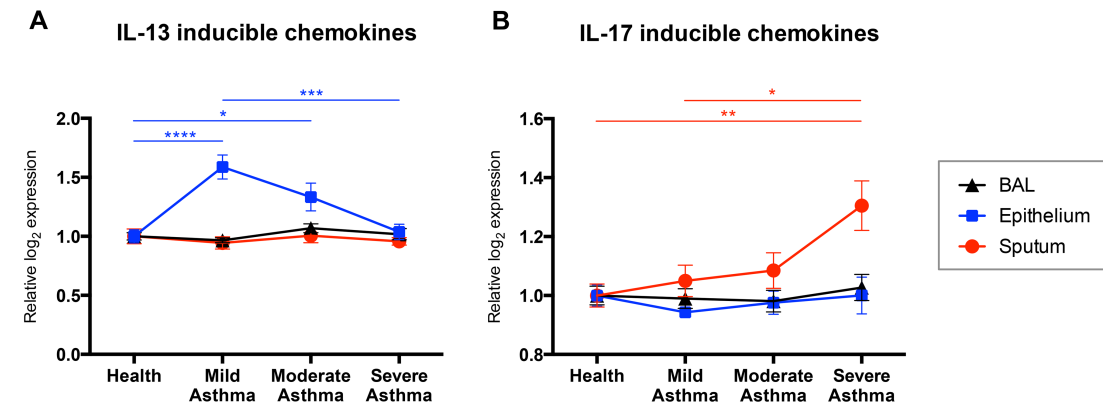
Figure 3.



Functional enrichment of combined differentially expressed gene list in severe asthma across all tissues. (A) Gene ontology analysis with arbitrary colours assigned to biological GO terms. *Defense response* and *immune system process* were assigned the same colour due to a large overlap in genes in these terms. GO terms with FDR corrected $P < 0.05$ were collapsed into categories of related terms using REVIGO, where $-\text{Log}_{10}$ p-values were considered significant. A red line indicating $P < 0.05$ 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 colour 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 colours reflecting their respective GO terms

694 from a). Toll-like receptor signaling KEGG pathway (FDR corrected $P < 0.05$) is
695 indicated with a dotted line.
696

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 colour of asterisks and bars representing the significance in the respective tissues. *, $P \leq .05$; **, $P \leq .01$; ***, $P \leq .001$; ****, $P \leq .0001$.