**Title:**  Innate and adaptive T cells in asthma: relationship to severity and therapy

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## Abstract

**Background**

Asthma is a chronic inflammatory disease involving diverse inflammatory cells and mediators whose interconnectivity and relationships to asthma severity are unclear.

**Objective**

A comprehensive assessment of T helper-17 (Th17), regulatory T-cells (Treg) and mucosal associated invariant T (MAIT)-cells in the context of other T-cell subsets and granulocyte mediators in asthma.

**Methods**

60 mild to severe asthmatics and 24 control participants underwent detailed clinical assessment and provided induced sputum, endobronchial biopsy, bronchoalveolar lavage and blood samples for analysis of adaptive and invariant T-cell subsets, cytokines, mast cell and basophil mediators.

**Results**

Significant heterogeneity of T-cell phenotypes was observed, with IL-13-secreting T-cells and type-2 cytokines increased in some but not all asthma severities. Th17-cells and γδ-17-cells, proposed drivers of neutrophilic inflammation, were not strongly associated with asthma, even in severe neutrophilic forms. MAIT-cell frequencies were strikingly reduced in both blood and lungs in relation to corticosteroid therapy and vitamin D levels, especially in severe asthma where bronchoalveolar lavage Treg were also reduced. Bayesian network analysis and topological data analysis identified complex interactions between pathobiologic and clinical parameters and novel clusters of underlying disease mechanisms, with elevated mast cell mediators in severe asthma, both in atopic and non-atopic forms.

**Conclusion**

The evidence for a role of Th17 cells in severe asthma is limited. Severe asthma is associated with a striking deficiency of MAIT cells and high mast cell mediator levels. This study provides proof of concept for disease mechanistic networks in asthma, with clusters that could inform the development of new therapies.

**Key messages**

• We provide proof of concept for a powerful new analytical approach to defining multi-dimensional clinical and pathobiologic clusters. This underlines the role of mast cells in two distinct subgroups of severe asthma characterized by the presence or absence of type-2 responses.

• The evidence for a role of Th17 cells in severe asthma is limited.

• We describe a striking deficiency of mucosal associated T cells as well as a mild reduction in regulatory T cells in severe asthma.

**Capsule summary**

This comprehensive analysis of airway T cell subsets and mast cells highlights the role of mast cells in severe asthma and demonstrates that novel mucosal associated invariant T cells are strikingly deficient in severe asthma.

**Key words**: Asthma, T-Lymphocytes, Cytokines, Mast cells, Phenotype, Endotype, Treg, Th17, Th2, Mucosal associated invariant T-cell

**Abbreviations**

ACQ 7 point Asthma Control Questionnaire

ANOVA Analysis of variance

BAL Bronchoalveolar lavage

BNA Bayesian network analysis

CD Cluster of differentiation

eNO Exhaled nitric oxide

FACS Fluorescence-activated cell sorting

GINA Global Initiative for Asthma

ICS Inhaled corticosteroid

IgE Immunoglobulin E

IL Interleukin

iNKT Invariant natural killer T

KS Kolmogorov-Smirnov

MAIT-cells Mucosal associated invariant T-cells

Tc Cytotoxic T-cell

TCR T cell receptor

TDA Topological data analysis

Th T helper

Treg Regulatory T-cell

## Introduction

Asthma is characterized by airways inflammation and remodeling. Based on initial studies in animal models1 and human T-cell clones,2 and bronchoscopy studies in mild steroid-naïve asthmatics,3,4 it has been viewed as a disease driven by activated T helper 2 (Th2)-cells producing type-2 interleukins (IL)-4, -5 and -13. These cytokines are believed to orchestrate the functions of mast cells,5,6 eosinophils7 and IgE-producing B cells/plasma cells. This concept has been challenged with increasing recognition of considerable heterogeneity of asthma mechanisms and definable patient subpopulations associated with immunopathology that cannot be explained by Th2 inflammation alone.8-11 Discoveries of novel T-cell subsets, notably anti-inflammatory regulatory T-cells (Tregs),12,13 and pro-inflammatory IL-17-secreting (Th17) T-cells14 and invariant natural killer T (iNKT)-cells,15 implicated in asthma pathogenesis on the basis of studies in animal models and limited evidence in human asthma, have added complexity to our understanding of immunoregulation. Recent studies have revived interest in mast cells,6,16 a subject of intense research in the 80s and early 90s, but not widely seen as candidate targets, possibly because of limited evidence of their role in severe disease.6,17,18

Most studies of asthma pathobiology to date have focused on individual or limited numbers of inflammatory cell types, making it difficult to appreciate the cell-cell interactions within complex networks that characterize inflammatory diseases like asthma. We have, therefore, undertaken a comprehensive assessment of bronchial and circulating T cells, including, mucosal associated invariant T (MAIT)19 cells, a novel cell type not yet studied in asthma or other chronic lung diseases, and relevant Th2/Th1/Th17 cytokines as well as mediators released in the lungs by mast cells and basophils. We have combined these pathobiologic findings with standard clinical parameters used to define asthma severity20 and interrogated the rich multi-dimensional clinico-pathobiologic dataset using the novel techniques of machine learning approach, i.e. Bayesian network analysis (BNA), and topological data analysis (TDA).21,22 This enabled an in-depth investigation of the roles of individual cell types in relation to asthma severity, characterization of the complex interconnectivity between the diverse clinical and pathobiologic parameters and identification of clinico-pathobiologic clusters which could point to novel asthma endotypes.

## Methods

### *Participants*

84 participants (18-70 years) were enrolled: 24 healthy non-atopic participants, 15 mild asthmatics on β2-agonists alone, 23 moderate asthmatics on inhaled corticosteroids (ICS) and 22 severe asthmatics with persistent symptoms despite high-dose ICS (n=16) and oral corticosteroids (n=6) (Table I and Figure E1, online repository) classified on enrolment applying criteria used previously15 (Table E1).

### *Study procedures*

Participants were assessed by history, examination, skin-prick testing with common aero-allergens, spirometry, exhaled nitric oxide (eNO), serum IgE and (except for severe asthmatics) methacholine responsiveness. Lung samples were obtained by sputum induction,23 bronchoalveolar lavage (BAL) and endobronchial biopsy.15,17 Using flow cytometry, the following T-cell subsets were characterized by their surface markers and intracellular cytokines15 in the circulation (blood) and lungs (sputum and BAL cell pellets; cells from collagenase-dispersed endobronchial biopsies to provide bronchial mucosal cells): Th17-, Th1- and IL-13-secreting Th2-cells, CD3+CD4+(or CD8-) FOXP3+ regulatory T cells (Tregs), cytotoxic T-cells (Tc)1 and Tc2, γδ-17 T-cells and MAIT-cells. Like other authors**24** we could not detect with confidence IL-4 or IL-5-secreting T-cells using intracellular cytokine staining. Type 1 (IFN-γ, IL-2, 12p70) and type-2 cytokines (IL-4, IL-5, IL-13), IL-10, IL-17A, mast cell proteases and basophil-derived basogranulin were measured in serum, sputum and BAL using standard methods (for details see online repository).

The study was approved by the Southampton and South West Hampshire Research Ethics Committee B. All participants provided informed consent.

### *Statistical analysis*

### Data elaboration and standard statistics

Data distribution was tested by the Shapiro-Wilk test and logarithmically transformed if not normally distributed. Groups were compared using Student’s *t* tests for two groups or, for multiple groups, one-way analysis of variance (ANOVA) with *post hoc* Dunnett’s (control as reference category). Groups ranked according to disease severity were tested for linear trend using polynomial contrasts. Where logarithmic transformation of zero values generated a loss of many data points, non-parametric tests were performed: Mann-Whitney-U (two groups), Kruskal-Wallis (multiple groups, with *post hoc* Dunn-Bonferroni), or pairwise Jonckheere-Terpstra test (linear trend across ranked groups).

### Network analyses

For network analyses (BNA and TDA) data were used from 62 participants with the most complete data. Missing data were imputed using average values specific to each tissue and disease severity subgroup. A composite value was generated for each parameter using a weighted average across each compartment: sputum and BAL for concentrations of soluble mediators and blood, sputum, BAL and biopsies for cell counts, providing airway and tissue composite readouts, respectively, with a matrix of 62 participants and 26 pathobiologic and 26 clinical parameters (See Tables E2, E3 for definitions of terms). Interconnectivity between clinical and pathobiologic parameters was first explored using BNA (Genie 2.0; Decision Systems Laboratory). Data were discretized to describe non-linear correlations into two bins (binary variables) or five to nine bins (continuous variables).

### Topological data analysis

To use the full range of available clinical and pathobiologic data simultaneously to identify multidimensional features within the dataset, which may not be apparent by traditional methods, we used the novel technique of topological data analysis (TDA), which is particularly suited to complex biological datasets. It combines features of standard clustering methodologies and also provides a geometric representation of the data21,22. In contrast to most other techniques which depend on prior hypotheses and which focus on pairwise relationships within the data25, this geometric visualization allows recognition of multi-dimensional features (patterns) within the data in a less supervised, data-driven, manner to identify meaningful subgroups that become apparent (self-define themselves) upon visualization (please see the TDA plots). In addition, TDA does not require *a priori* definition of the number of clusters anticipated.

TDA was performed as described21 using IRIS 2.0 (Ayasdi Inc.), constructing networks with parameters from Table E3. Three inputs were used: a distance metric, one or more filter functions and two resolution parameters (“resolution” and “per cent overlap” or “gain”). A network of nodes with edges between them was created using a force directed algorithm. The nodes represent bins or “micro-clusters” of data points, and two nodes are connected if their corresponding collections of data points have a point in common.21 Variance normalized Euclidean distance was used as a distance-metric and two filter functions: principal and secondary metric singular value decomposition (further explanations in online repository). Resolution and gain settings were selected for which the network structure permits identification of subgroups. Kolmogorov-Smirnov (K-S) tests identified parameters which differentiate each subgroup from the rest of the structure and create clusters. Comparisons between multiple clusters used one-way analysis of variance (ANOVA), with *post-hoc* tests with Bonferroni adjustment for multiple comparisons.

For additional methods see online repository.

## Results

Data were first analyzed by standard statistical methods, without using imputation or composite averages, classifying subjects as healthy, mild, moderate or severe asthmatics. Previous observations that mild, steroid-naïve asthma is characterized by a bias towards type-2 inflammation were confirmed, with increased numbers of IL-13-secreting CD4+ (Th2) cells in mild asthmatics in sputum, BAL and endobronchial biopsies (Figure 1A) and ratios of IL-13 to IFN-γ-secreting CD4+ (Th1) cells (Figure E2). However, this bias was not seen in severe asthma, where frequencies of IL-13-secreting Th2 cells were not significantly different from health, although we did not measure frequencies of IL-4 or IL-5-secreting T cells. Similarly, in mild asthma there were significant increases in concentrations of type-2 cytokines, IL-5 (P<0.001) and IL-13 (P<0.05) in BAL (Figure E3). In sputum, neither IL-5 nor IL-13 were elevated in mild or moderate asthma. In severe asthma, IL-5 was also significantly elevated in both BAL (P<0.05) and sputum (P=0.005) (Figure E3B), suggesting that its secretion may be relatively steroid-insensitive and may be derived from cellular sources other than airway Th2 cells.

Analysis showed no significant differences in Th17-cell or γδ-17 T-cell frequencies between asthma and health (Figures 1B, Figure E6). Furthermore, we found no evidence of dysregulation of the Th17 response during cold-induced exacerbations of asthma (see online repository). Likewise, IL-17 was not increased in asthma in BAL, sputum or serum (Figure E3B and data not shown) (P>0.05 in all compartments). However, when asthma was stratified according to severity, IL-17 in BAL was increased in mild asthmatics (P=0.04, Figure E3A); levels were associated with allergic rhinitis (P=0.02), airway eosinophilia (*r*s=0.34, P=0.04) and high serum IgE (*r*s=0.42, P=0.007) (Figure E3C).

Th17 cells share functionally and developmentally antagonistic relationships with the immunoregulatory Tregs which regulate airway hyperresponsiveness in murine asthma models.26 We observed a slight, but significant, deficiency of BAL CD4+FOXP3+ Tregs in asthma (median frequency 5.3%, IQR 4.3-8.2%) compared with health (8.1%, 5.6-10%, P=0.03, Figure 1C) which was restricted to severe asthmatics (Figure 1C) and was not evident in blood, sputum or bronchial biopsies.

There was a striking deficiency of TCR-Vα7.2+CD161+ MAIT-cells in asthma in blood, sputum and biopsies, which was related to disease severity and treatment with inhaled corticosteroids (Figure 2, Figure E4). There was evidence of seasonal variation in MAIT-cell frequencies and association with serum vitamin-D3 concentrations and use of oral corticosteroids (see online repository).

### Network Analyses

Application of Bayesian network analysis to pathobiologic and clinical features in relation to asthma severity showed complex, non-linear associations (Figure 3). Five nodes (eNO, IL-17, IFN-γ, neutrophils and vitamin D) without strong interactions with other parameters were not connected and, therefore, remained outside the network. The asthma severity node was strongly connected with mast cell mediators, tryptase, chymase and carboxypeptidase A3, and with IL-13- and IFN-γ-secreting CD8+ cytotoxic T-cells (Tc2 and Tc1, respectively). There was a strong negative association between MAIT-cell frequencies and ICS use which, in turn, was positively associated with asthma severity.

To look further for novel associations between clinical and pathobiologic features, we applied topological data analysis (TDA) to all acquired clinical and pathobiologic data (Figures 4, 5, Table E4). Data were treated as composite averages for T-cell subsets (across blood, sputum, BAL and biopsies), cytokines and eosinophils (across blood, sputum, BAL), neutrophils, macrophages, lymphocytes, mast cell mediators, basogranulin (across sputum and BAL). One healthy and six asthma clusters were identified.

The TDA-derived Cluster-1, comprising predominantly mild, atopic asthmatics, had (compared to other asthmatics) better lung function, lower asthma control questionnaire (ACQ)27 score (mean 0.88) and severity, as assessed by physician on enrolment or Global INitiative for Asthma (GINA) criteria,20 and patients were mostly not on ICS. They had elevated IL-13-secreting Th2-cells, lower IL-13 and tryptase and were predominantly paucigranulocytic (sputum neutrophils ≤61%, eosinophils ≤3%) (Figure 4B, Table E4).

Cluster-2 consisted of well-controlled asthmatics (mean ACQ 0·5), with little evidence of inflammation (the only abnormality being eosinophilia) and lower frequencies of Tregs and IFN-γ secreting CD8+ T-cells (Figure 4).

Cluster-3 consisted of moderately severe (defined by enrolment criteria) and partially controlled asthmatics (by GINA criteria20) despite ICS; they had the highest bronchodilator reversibility and eNO levels. Their pathobiologic profile consisted of type-2 inflammation with, highest levels of IL-5 and IL-13 and high frequencies of IL-13-secreting Th2-cells in bronchial biopsies (Figures 5A,E11,E13) but also other T-cell subsets, Th1, Th17, Tregs.

Cluster-4 was a small group with later-onset disease (mean 28 yr), moderately severe by physician assessment, nasal polyposis and salicylate-sensitivity, low IL-17.

Cluster-5 asthmatics were older (mean age 50 yr), with high BMI (mean 32.6), poor lung function, high symptoms (mean ACQ 2.1) and high treatment requirements (predominantly GINA step 4/5 and mean 1500 mcg/day of beclometasone dipropionate equivalent). Their pathobiologic profile was high type-2 cytokines (IL-5 and IL-13), IL-13 secreting CD8+ T (Tc2)-cells (Figure E14D) and high tryptase, chymase and carboxypeptidase A3 (Figures 4, 5C, E10, E11). However, when compared with cluster-3 (also type-2 cytokine-high), cluster-5 had fewer Tregs and higher ICS use (mean difference 1250 mcg/day).

Cluster-6 was predominantly female, obese (mean BMI 35), non-atopic, with salicylate-sensitivity, later-onset (mean age 25 yr) and were the most severe cluster by GINA classification, physician assessment, symptoms (mean ACQ 3.2) and lung function (mean pre-bronchodilator FEV1 62%), despite high dose ICS (Figure E12B) and, frequently (50% of group), maintenance oral corticosteroids (mean 14 mg prednisolone/day). Their key pathobiologic features were high carboxypeptidase A3 and a profound MAIT-cell deficiency (Figures 4E, 5D, E11D). They also had low Tc1, Th17, and IL-13-secreting Th2-cells (Figure E11A) but elevated tryptase (Figure 4E,5C) and chymase. This cluster contained a higher proportion of participants who could be classified as cluster 5 described by the Severe Asthma Research Program (SARP)9 (Figure E14B) and the obese, non-eosinophilic cluster reported by Haldar *et al.*10

The majority of participants with sputum neutrophilia, defined as neutrophils >61%28 were in clusters 5 and 6, while eosinophilic asthma was distributed across clusters 1, 2, 3 and 5 but not 4 or 6 (Figure 4B).

## Discussion

Asthma is a common disease whose clinical severity ranges from mild forms, controlled with β2-agonists alone or low doses of inhaled corticosteroids, to very severe, requiring high doses of inhaled and oral corticosteroids and, increasingly, biologics such as the anti-IgE monoclonal antibody, omalizumab.29 In this study comprehensive analysis of T-cells, granulocytes, cytokines and mast cell mediators across the airway lumen, mucosa and blood compartments pointed to their relative roles within the asthma syndrome that have not been recognized before: reduced MAIT-cell frequencies as a striking feature that is related to asthma severity, reduced Treg frequencies in severe disease and raised mast cell mediators in severe disease, consistent with corticosteroid-insensitive mast cell activation. This study shows that the asthma spectrum can be broken down into several multi-dimensional clusters defined by combined clinical parameters and underlying mechanisms (pathobiology), which provides proof of concept for endotyping asthma for better understanding of its mechanisms and more focused drug development.

Original descriptions of asthma pathobiology3 suggested a key role for Th2-mechanisms. Consistent with this concept, when all the asthmatics in this study were compared as a group with healthy participants, the most significant asthma discriminators were airway eosinophilia, higher mast cell mediators (carboxypeptidase A3, chymase and tryptase), IL-5 and -13, eNO and serum IgE, but lower IFN-γ, a pathobiologic profile classically associated with Th2-inflammation (Figure 1A, E10). The application of BNA showed high connectivity between the asthma severity node and nodes for mast cell mediators and IL-13- and IFN-γ-secreting CD8+ cytotoxic T-cells (Tc2 and Tc1, respectively) and a strong negative association between MAIT-cell frequencies and asthma severity and ICS consumption. Applying the recently developed TDA method21,22 to the same dataset showed complex, multi-dimensional clusters, i.e. possible endotypes defined by a combination of clinico-pathobiologic features.8,11 The advantage of TDA over standard clustering methodologies is that it provides geometric representations of complex and multi-dimensional datasets which reveal and stratify distinct subgroups.22 It combines features of standard statistical methods, such as singular value decompositions and similarity metrics, to construct a network that clusters most similar data-points into nodes. A node in a TDA network represents a group of most similar data-points (in this case two or more subjects which are similar in multiple dimensions). Each node can be joined to the next node if the nodes share common data-points (i.e. subjects). This allows a natural continuous network when the phenomenon is not disjoint.21 TDA can deal with both linear and non-linear associations and identifies significant subgroups in a data-driven manner, allowing for finer stratification.21 Furthermore, TDA is sensitive to both large and small scale patterns that other techniques, such as, clustering and multidimensional scaling, often fail to detect, as they sometimes obscure geometric features captured by topological methods. Hierarchical clustering cannot easily identify these subgroups as it tends to separate points which may in fact be close in the data.21

The finding of clinico-pathobiologic clusters in the dataset in this study should improve our understanding of asthma and inform drug development. Over-expression of the Th2-cytokine network in cluster-1 is similar to the original reports in corticosteroid-naive asthmatics highlighting the role of type-2 mechanisms in asthma.3 Clusters 3 and 5 share many clinical features, including atopy, allergic rhinitis and emotion-related symptoms (see online repository). Both are characterized by type-2 inflammation, with the highest levels of IL-5, IL-13 and IL-10, suggesting that these clusters reflect asthma endotypes that may be particularly suitable for biologics such as mepolizumab30 and lebrikizumab,31 which currently use indirect biomarkers, eosinophil counts and serum periostin, to select patients to maximize clinical efficacy. However, important differences between these two clusters were identified: higher Treg frequencies in cluster-3 might explain their lower corticosteroid requirements, whilst lower IL-13-secreting Th2-cell frequencies and higher tryptase in cluster-5 suggest distinct, steroid-insensitive mechanisms.32 It should be noted that we stained only for IL-13, so we cannot exclude an increase in Th2-cells secreting IL-4 or IL-5 in the more severe asthma clusters. We observed some differences between patterns of cytokine secretion in sputum and BAL, which may arise because BAL samples the distal airways and alveoli whilst sputum reflects changes in more proximal airways.33

The strong association between asthma severity and mast cell mediators in clusters 5 and 6 suggests that severe asthma is a disease in which mast cell activation plays an important role. Our data add to evidence implicating mast cells in severe asthma, providing additional confirmation which should stimulate development of drugs that target mast cells. Brightling et al have described increased numbers of tryptase positive mast cells infiltrating the airway smooth muscle in mild 6 and severe34 asthma in numbers that correlate with airway hyper-responsiveness.6,18 In the Severe Asthma Research Program, Balzar *et al* reported that severe asthma was associated with an increase in bronchial mast cells staining positive for both tryptase and chymase and with BAL concentrations of PGD2, a lipid mediator associated with mast cells and shown to increase after allergen challenge.5,16

The current study adds to the evidence5,16,35 that mast cell activation is insensitive to corticosteroids and suggests that severe asthma, where mast cell mediators are elevated, can be stratified further by clinical features, such as atopic status, and also by evidence of type-2 cytokine mediated mechanisms in cluster-5 but not cluster-6. We speculate that anti-IgE antibody omalizumab might exhibit some of its beneficial effect in severe atopic asthma via inhibition of IgE-mediated, mast cell activation. This finding may yield a prognostic biomarker for this biologic, which is currently missing, and may extend the indication for omalizumab to non-atopic asthma, where a preliminary trial has suggested clinical efficacy.36

Several asthma studies have reported raised IL-17,37,38 but this study found only limited evidence for Th17 cells and none for γδ-17-cells, either during a period of clinical stability or during an exacerbation. This is consistent with the findings of a recent trial in which the anti-IL17 receptor A monoclonal antibody, brodalumab, had no effect on symptoms or lung function in moderate to severe asthmatics.39 We did, however, observe associations between IL-17 concentrations and traditional type-2 biomarkers (airways eosinophils and serum IgE) which have not been reported before as IL-17 is mainly been implicated in neutrophilic inflammation in asthma.40 Our study also identified, for the first time, reduced numbers of CD3+4+FOXP3+ ‘Treg’s in severe asthma. In humans some up-regulation of the nuclear transcription factor FOXP3 has been observed in non-suppressive T cells upon TCR stimulation.41 Although we were not able to further validate the identity of these FOXP3+ T-helper cells as T reg with additional surface markers, we observed low rates of spontaneous T cell activation, suggesting that activated T-cells will comprise only a small proportion of the CD3+4+FOXP+ T-cells enumerated.

An important finding in this study is the striking deficiency of MAIT-cells in both the circulation and lungs which correlated strongly with clinical severity. To our knowledge, MAIT-cells have not yet been studied in any airways disease. This study suggests that they are more abundant than iNKT-cells,15 comprising up to 10% of blood and airway T-cells. Their marked evolutionary conservation implies an important role in immunity.42,43 MAIT-cells are the most abundant T-cell subset able to detect and kill bacteria infected cells. Recent animal models of bacterial airways infection indicate their critical role in lung host defense.42,44 We found MAIT-cell frequencies to be associated with serum vitamin D3 concentrations and, in pilot data, could be suppressed by one week of treatment with prednisolone (online repository and Figure E9). The lack of a significant deficiency of MAIT-cells in BAL may result from low peripheral deposition of ICS in the more distal airways and alveolar compartments sampled by lavage.33 Their deficiency in severe asthma, whether primary or resulting from chronic corticosteroid use, may contribute to increased susceptibility to bacterial infection recognized in severe asthma45,46 and to changes in the airway microbiome,47 and may thus impact on asthma pathology.44

In summary, this study sheds light on previously unreported observations in asthma in relation to disease severity. The observation of clusters composed of clinical and pathobiologic parameters will need to be reproduced before these clusters can be accepted as novel endotypes of asthma. However this paves the way for future asthma studies in large patient cohorts, such as SARP9 and U-BIOPRED,48 where distinct asthma endotypes could be identified and subsequently validated, allowing translation to clinical trials and routine clinical practice.

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## Table I. Clinical characteristics of participants

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | **Healthy controls** | | **Mild asthma** | | **Moderate asthma** | | **Severe asthma** | |
| *n* |  |  | 24 |  | 15 |  | 23 |  | 22 |  |
| Demographics | | |  |  |  |  |  |  |  |  |
| Sex (M/F) | |  | 14 / | 10 | 8 / | 7 | 10 / | 13 | 8 / | 14 |
| Age (median [range], years) | | | 28 | (20-65) | 26 | (21-64) | 36 | (21-56) | 53 | (23-67) |
| Pulmonary function | | |  |  |  |  |  |  |  |  |
|  | FEV1 (% predicted) | | 108 | (105-113) | 88 | (86-103) | 99 | (86-107) | 65 | (49-82) |
|  | FEV1 reversibility (%) | | 3.3 | (1.8-7.4) | 13 | (11-19) | 10 | (2.2-17) | 13 | (2.6-25) |
|  | PEFR (% predicted) | | 108 | (97-116) | 98 | (89-107) | 95 | (85-100) | 70 | (53-82) |
|  | PEFR variability (%) | | 0 | (0-11) | 17 | (10-25) | 22 | (17-32) | 17 | (12-24) |
|  | PD20 (mg methacholine) | | Negative | | 0.19 | (0.050-0.79) | 0.25 | (0.063-0.73) | Not done | |
| Exhaled nitric oxide (ppb, at 50 L/s) | | | 16 | (11-21) | 53 | (27-107) | 26 | (15-51) | 20 | (13-38) |
| Clinical | |  |  |  |  |  |  |  |  |  |
| Atopy (Skin test positive, Y/N) | | | 0 / | 24 | 15 / | 0 | 20 / | 3 | 15 / | 7 |
|  | No. of skin test allergens positive | | 0 | (N/A) | 6 | (4-7) | 3 | (2-5) | 3.5 | (0-5.3) |
|  |  |  |  |  |  |  |  |  |
| Peripheral eosinophil count (109/L) | | | 0.1 | (0.1-0·2) | 0.2 | (0.1-0.6) | 0.2 | (0.15-0.3) | 0.2 | (0.1-0.3) |
| Total IgE (IU/ml) | | | 26 | (10-61) | 172 | (21-451) | 105 | (35-188) | 84 | (31-669) |
| Body mass index (kg/m2) | | | 24.4 | (22.5-28.1) | 23.6 | (22.7-26.5) | 25.3 | (23.3-30.9) | 31.0 | (27.1-40.9) |
| Smoking status | | |  |  |  |  |  |  |  |  |
|  | Never |  | 21 |  | 14 |  | 19 |  | 17 |  |
|  | Former | (Mean pack-years) | 3 | (4·2) | 1 | (6·7) | 4 | (5·8) | 4 | (26) |
|  | Current | (Mean pack-years) | 0 |  | 0 |  | 0 |  | 1 | (49) |
| Duration of asthma (years) | | | N/A |  | 18 | (15-26) | 22 | (9-27) | 36 | (21-49) |
| ACQ score | |  | N/A |  | 0.60 | (0.43-1.3) | 1.0 | (0.60-1.4) | 2.8 | (2.2-3.5) |
| GINA level of control (n, %) | | |  |  |  |  |  |  |  |  |
|  | Controlled | | N/A |  | 8 | (53) | 5 | (22) | 0 | (0) |
|  | Partly controlled | | N/A |  | 6 | (40) | 15 | (65) | 2 | (9.5) |
|  | Uncontrolled | | N/A |  | 1 | (6·7) | 3 | (13) | 19 | (90) |
| Treatment | |  |  |  |  |  |  |  |  |  |
| Inhaled steroids | | | No |  | No |  | Yes |  | Yes |  |
|  | Dose (equivalent mcg BDP) | | N/A |  | N/A |  | 400 | (200-400) | 1600 | (1280-2000) |
| Maintenance oral corticosteroids (Y/N) | | | No |  | No |  | No |  | 6 / | 16 |
|  | Mean dose if taken (mg prednisolone/day) | |  |  |  |  |  |  | 11 |  |
| Short acting β agonist (Y/N) | | | No |  | Yes |  | Yes |  | Yes |  |
| Long acting β agonist (Y/N) | | | No |  | No |  | 10 / | 13 | 22 / | 0 |
| Leukotriene receptor antagonist (Y/N) | | | No |  | No |  | 1 / | 22 | 15 / | 7 |
| Step on GINA treatment algorithm | | | N/A |  | 1 |  | 2 - | 3 | 4 - | 5 |
|  |  |  |  |  |  |  |  |  |  |  |
| Inflammatory subtype (n, %) | | |  |  |  |  |  |  |  |  |
|  | Neutrophilic | | 4 | (25) | 2 | (15) | 2 | (11) | 10 | (48) |
|  | Eosinophilic | | 1 | (6.3) | 3 | (23) | 3 | (17) | 6 | (29) |
|  | Mixed granulocytic | | 0 | (0) | 0 | (0) | 0 | (0) | 1 | (4.8) |
|  | Paucigranulocytic | | 11 | (69) | 8 | (62) | 13 | (72) | 4 | (19) |
| Sputum cell differential (%) | | |  |  |  |  |  |  |  |  |
|  | Macrophages | | 52 | (31-66) | 49 | (35-64) | 47 | (30-62) | 30 | (19-43) |
|  | Neutrophils | | 31 | (11-65) | 34 | (22-54) | 33 | (16-56) | 61 | (32-76) |
|  | Epithelial | | 3.6 | (2.0-24) | 4.3 | (1.7-10) | 4.1 | (1.1-21) | 2.9 | (0-7.8) |
|  | Eosinophils | | 0.38 | (0-0.94) | 1.5 | (0·75-1.8) | 0.75 | (0.25-1.5) | 0.69 | (0-6.1) |
|  | Lymphocytes | | 0.1 | (0-0.75) | 0.3 | (0-0.75) | 0 | (0-0.68) | 0.0 | (0-0.25) |
| BAL cell differential (%) | | |  |  |  |  |  |  |  |  |
|  | Macrophages | | 84 | (74-89) | 70 | (60-80) | 81 | (73-89) | 72 | (46-94) |
|  | Neutrophils | | 2.5 | (1.0-5.9) | 2.5 | (1.6-4.8) | 3.5 | (1.8-6.4) | 6.5 | (1.4-29) |
|  | Epithelial | | 9.9 | (3.9-18) | 21 | (13-35) | 11 | (5.6-19) | 8.7 | (3.3-11) |
|  | Eosinophils | | 0.25 | (0.0-0.56) | 2.0 | (0.75-3.6) | 1.0 | (0-3.0) | 0.1 | (0-1.6) |
|  | Lymphocytes | | 1.4 | (0.94-2.4) | 1.5 | (0.38-3.0) | 1.3 | (0.5-2.3) | 1 | (0-1.6) |
| Relevant comorbidities (n, %) | | |  |  |  |  |  |  |  |  |
|  | Allergic rhinitis | | 0 | (0) | 12 | (80) | 11 | (58) | 10 | (46) |
|  | Nasal Polyps | | 0 | (0) | 0 | (0) | 1 | (5.3) | 5 | (23) |
|  | Eczema |  | 3 | (13) | 7 | (47) | 6 | (32) | 4 | (19) |
|  | Bronchiectasis | | 0 | (0) | 0 | (0) | 1 | (5.3) | 1 | (4.5) |
|  | | | | | | | | | | |

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. ACQ, asthma control questionnaire;27 BDP, beclometasone dipropionate; CT, computed tomograph; FEV1, pre-bronchodilator forced expiratory volume in 1 second; FVC, forced vital capacity; GINA, Global Initiative for Asthma;20 mcg, micrograms; mg, milligrams; PEFR, peak expiratory flow rate; PD20, provocative dose causing a 20% fall in FEV1.

## **Figure legends**

**Figure 1.**

(A) Frequencies of CD3+CD4+ T-cells expressing IL-13 (Th2 cells), (B) CD3+CD4+ T-cells expressing IL-17 (Th17 cells) and (C) FOXP3 (Treg) in PBMC, sputum, BAL and bronchial biopsies, as percentage of live CD3+CD4+ T cells or, for endobronchial biopsies, percentage of CD3+CD8- T cells. Horizontal lines show medians. Left columns: healthy controls versus asthmatics, with Mann-Whitney U P values. Right three columns: stratified by disease severity, with Kruskal-Wallis P values where P<0.05. Significance *post hoc* by Dunn’s test compared with health: \*P<0.05, \*\* P<0.01. Abbreviations: BAL, bronchoalveolar lavage; Mod, moderate; PBMC, peripheral blood mononuclear cells.

**Figure 2.**

MAIT-cells (Vα7.2+CD161+) as proportions of CD3+ T-cells) in blood, sputum, BAL and endobronchial biopsies (A) in health and asthma, and (B) stratified by disease severity. Horizontal lines show medians. Unpaired t tests on log-transformed data. MAIT cell deficiency correlates with severity by linear trends across groups using residuals on log-transformed data (given where P<0.05). *Post hoc* Dunnett’s test compared with health: \* P<0.05, \*\* P<0.01.

**Figure 3.**

Bayesian Belief Network showing strongest interactions between pathobiologic parametersacross a range of clinical severities of asthma or health. Nodes without strong interactions are excluded. Line thickness represents strength of interaction (Euclidean distance). Line colors: green, positive associations; red, negative associations; black, non-linear associations. Asthma severity: overall physician assessment at enrolment (see Table E1). Abbreviations: BMI, body mass index; ICS, inhaled corticosteroids; IL, interleukin; MAIT, mucosal associated invariant T-cell; Tc1, CD8+IFN-γ+ T cell; Tc2 CD8+IL-13+ T cell; Th, T helper cell; Treg, regulatory T-cell.

**Figure 4.**

Panel A. Multi-dimensional clinico-pathobiologic clusters in asthma and health. Topological network analysis of clinical and pathobiologic features generates one healthy (blue) and six distinct clinico-pathobiologic asthma clusters (1-6). The network is colored by disease severity (GINA classification), with most severe subjects in red and the other, milder forms in varying shades of orange, yellow and green.

Panel B shows the same network as Figure 4 overlaid with distribution of neutrophilic (sputum neutrophils >61%, green) or eosinophilic (sputum eosinophils >3%, red) asthma. Panel C shows the frequencies of MAIT-cells. Panel D shows the network colored by average concentrations of the type-2 cytokines, IL-4, IL-5 and IL-13 in serum, sputum and BAL. Panel E: the network is colored by concentrations of mast cell tryptase in sputum and BAL. In panels B-E the colors represent concentrations or frequencies, ranging from low (blue) to high (red) concentrations.

The TDA used 62 subjects with most complete data. The metric used is variance normalized Euclidean. The lenses used were principal and secondary singular value decomposition (SVD)(Resolution 32, Gain 4.0/3.5x, Equalized); Node size: proportional to number of individuals in node.

**Figure 5.**

Analyses generated from the clinico-pathobiologic TDA network in Figure 4 show concentrations of interleukin-5 (A) averaged across serum, sputum and BAL, (B) Exhaled nitric oxide concentrations (C) Mast cell tryptase in BAL and sputum, (D) MAIT-cells in blood, sputum, BAL and bronchial biopsy. Box and whisker plots show medians, interquartile ranges and ranges. Statistical tests: one way ANOVA, with *post hoc t* tests compared with health, using Bonferroni correction (\*P<0.05, \*\* P<0.01, \*\*\* P<0.001).