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Article

Activation status of airway immune cells is a defining feature of severe asthma, regardless of fungal sensitisation

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ABSTRACT

Airborne fungi are potent inducers of respiratory disease and cause the debilitating conditions severe asthma with fungal sensitisation (SAFS) and allergic bronchopulmonary aspergillosis (ABPA). However, the immune cell types and the inflammatory airway environment that defines SAFS and ABPA patients is not extensively characterised. To address this, we recruited SAFS and ABPA patients, asthmatics without evidence of fungal sensitisation and healthy controls (n = 20 individuals per group). Immune cells were isolated from collected sputum and peripheral blood samples and immunophenotyping was performed via flow cytometry. By applying a machine learning approach to our dataset, we identify a critical association between $CD4^+$ T cells, type 2 conventional dendritic cells, eosinophils, proinflammatory factors and severe respiratory disease. These complex immune signatures should be investigated further to improve the diagnostics and treatment of SAFS and ABPA.

Introduction

Global estimates of asthma prevalence are rising, with an estimated 262 million affected individuals in 2019.[1] Purported reasons for this rapid rise include urbanisation of lower- and middle-income countries resulting in higher rates of obesity, poorer air quality, diminished early exposure to pathogens, and improved asthma diagnostics.[2–4] For most asthmatics, symptoms can be controlled with inhaled therapies. However, in 10 % of patients (severe asthma SA patients), high doses of inhaled corticosteroid (ICS) and additional controller medications are required to prevent exacerbations. Even then, their asthma may remain poorly controlled.[5–6] Variation in treatment response can be attributed in part to the different types of inflammation which underpin asthma. Between 70–80 % of patients have type 2 (T2) inflammation which is characterised by eosinophilia and the T2 cytokines IL-4, IL-5 and IL-13.[7–11] These patients are generally responsive to oral corticosteroids (OCS) and novel biologic therapies such as Benralizumab (anti-IL-5 receptor), Mepolizumab and Reslizumab (anti-IL-5), Omalizumab (anti-IgE), or Dupilumab (anti-IL-4/IL-13 receptor).[11] However, a significant proportion of patients have T2 'low' asthma, the majority of whom experience airway neutrophilia and elevated levels of IL-8, IL-17 and IL-22. These patients are often less responsive to conventional treatments and less is known about this form of the disease.[9–10,12]

Fungi such as *Aspergillus fumigatus (Af), Alternaria alternata* and *Cladosporium herbarum,* can be potent inducers and exacerbators of respiratory disease.[13–17] In severe asthma, 30–50 % of patients are

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sensitive to fungi, and this is strongly correlated with poor treatment response, reduced lung function, increased hospitalisation and asthma-related deaths.[16,18] Clinically, this is either described as severe asthma with fungal sensation (SAFS) or allergic bronchopulmonary aspergillosis (ABPA).[19–20] Despite affecting 10 million people worldwide, SAFS and ABPA remain challenging to diagnose and treat.[14,21] The current diagnostic criteria for SAFS are 1) the presence of severe asthma, 2) elevated serum total IgE (<1000 IU/mL) 3) elevated specific IgE (>0.35kU/L) to fungal allergens and/or a positive skin-prick to test to fungal allergens 4) the absence of clinical/radiological features of bronchiectasis. For ABPA diagnosis, total IgE must exceed 500 IU/mL and two additional criteria are required. Such include radiological evidence of bronchiectasis, blood eosinophilia > 0.5 x 10^9/L, or positive *Aspergillus* precipitins/IgG.[19–20] However, it is likely that a large proportion of ABPA patients, particularly in the developing world, are misdiagnosed and treated for recurrent bacterial infections.[22–23] Therefore, it is imperative to improve diagnostic testing to allow subsequent appropriate treatment strategies for SAFS and ABPA.

Much of our mechanistic understanding of fungal sensitisation in the lung comes from murine models.[17,24] Our group has recently shown that dendritic cell (DC) recognition of early growth stages of *Af* spores plays an important role in T cell mediated allergic airway inflammation, with the capacity to induce both eosinophilia and neutrophilia.[17] However, whether DCs and T cells are important contributors in driving fungal asthma in humans is poorly understood.

To better understand the events that underpin SAFS and ABPA, we characterised immune cell populations of sputum and matched blood samples from a large asthma cohort. Despite the technical challenges in isolating immune cells from sputum, we successfully utilised multi-parameter flow cytometry to identify the proportion and activation status of granulocytes, myeloid and T cell populations. With the application of machine learning approaches on cellular data, we found that severe asthma groups (regardless of fungal sensitisation) clustered separately from mild asthma (MA) and healthy controls (HC). However, the cellular parameters which were most informative in clustering patients varied between sputum and blood. Clustering from blood samples was based largely on the abundance and activation of $CD4^+$ T cells. In contrast, clustering from sputum samples was informed by $CD4^+$ T cells, DC subsets (particularly type 2 conventional dendritic cells (cDC2s)) and eosinophils. Furthermore, we found that the concentration of IFNγ, IL-9 and eotaxin in sputum supernatants were also reliable indicators of asthma severity. These results show the importance of charactering the airway environment of asthma patients to better understand the immune events which cause disease. They further highlight $CD4^+$ T cells and cDC2s as critical players in severe respiratory disease and potential risk factors for fungal sensitisation.

Results

The immune events that occur in the airways of asthma patients, particularly those who are fungal sensitised, are poorly understood.[21] To redress this, we isolated cells from the sputum and blood of HC, MA, SA, SAFS and ABPA patients (Fig. 1A).

The baseline characteristics of the study cohort are described in Table 1. Median age was 52 years (19–78 years) and mean body mass index was 29.1 ± 7.7 (kg/m^2). Most participants were White British (65 %) and never-smokers (57 %). Available lung function data from our severe disease groups demonstrated significant airway obstruction. The mean forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) ratio in SA, SAFS and ABPA patients was 0.65 ± 0.12 to 0.63 ± 0.14 and 0.58 ± 0.13, compared to 0.84 ± 0.03 in MA.

With recruitment taking place in 2018, this cohort would not have received recently approved biologic interventions such as Benralizumab, Mepolizumab and Dupilumab to control their asthma. Most patients were being treated with high dose ICS and additional controller medications. However, within the SA, SAFS and ABPA groups 11 %, 33 % and 5 % of patients were taking the anti-IgE medication Omalizumab. Furthermore, whilst 47 % of the SA cohort had been previously prescribed the macrolide antibiotic Azithromycin, there was greater use of anti-fungals in the SAFS and ABPA patients (Table 1).

The frequency of granulocytes, myeloid and T cell populations differ between sputum and blood, regardless of disease

Using a 26-parameter flow cytometry panel, we identified granulocytes, myeloid, and T cell populations in both sputum and blood samples (Fig. 1B, Supplementary Fig. 1 & 2, Supplementary Table 1). Following removal of highly autofluorescent cells (likely macrophages),[25] the majority of $CD45^+$ cells in the sputum were granulocytes (Fig. 1B). Though neutrophils dominated this compartment (Fig. 1C), eosinophils were also detected (Fig. 1C). In the blood, there was a more equal representation of granulocytes, T cells and myeloid cells (Fig. 1B).

Within the myeloid and lymphoid families, we then identified classical, intermediate and non-classical monocytes, conventional DC and monocyte-derived DC subsets (Fig. 1D), as well as γδ, $CD4^+$, $CD8^+$ and regulatory T cell populations (Fig. 1E). Regardless of patient group, the proportion of these T cell populations differed greatly between the sputum and blood (Fig. 1D & E, Supplementary Table 2). For example, δγ cells were abundant in the blood but largely absent from the sputum (Fig. 1E).

Having determined the frequency of these immune cell populations in both sputum and blood, the expression of various surface markers in key populations was assessed to elucidate function and activation state (Supplementary Table 3).

Phenotyping immune cells in the blood identifies individuals with severe asthma

To determine whether the flow cytometry data from peripheral immune cells was significantly different between disease conditions we first applied multi-variate statistical analysis. We utilised 31 parameters relating to the blood which described immune cell population frequency (measured as both percentages of total $CD45^+$ cells and the direct parent population) and expression of markers associated with activation in certain immune cell populations (Supplementary Table 3 & 4). Clinical co-variates and data obtained from the sputum were not included. After correcting for the false discovery rate, there were no significant differences between the different cohorts by Permutational MANOVA (Fig. 2A). Similarly, principle component analysis (PCA) did not reveal evident clustering of disease groups (Supplementary Fig. 3A).

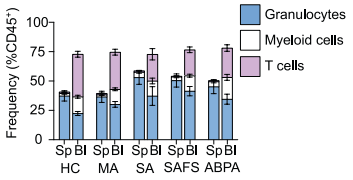
To interrogate our data further, we wanted to test whether features of the peripheral blood immune phenotype could predict disease condition by building a random forests (RF) model. This is a supervised machine learning method that can incorporate large numbers of variables to classify samples using randomly generated decision trees.[26] These decision trees can be used to cluster samples and interrogate the relative importance of variables within the dataset. They can also estimate their own error rate via bootstrapping. Our RF and multidimensional scaling (MDS) analysis revealed evident clustering of HC and MA patients ('non-severe' cluster) versus SA, SAFS & ABPA patients ('severe' cluster) (Fig. 2B). The model was able to correctly classify patients into these non-severe and severe groups with an average error rate of 20 % (Supplementary Fig. 4A).

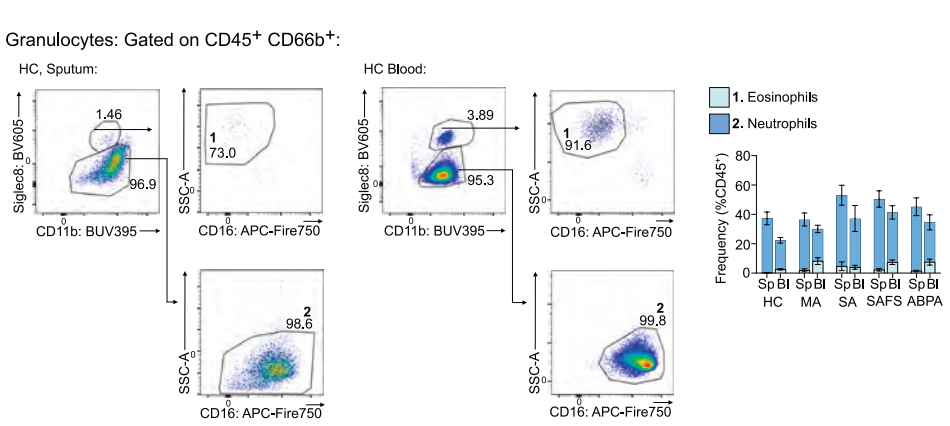
Peripheral T cells are critical for identifying individuals with severe airway disease

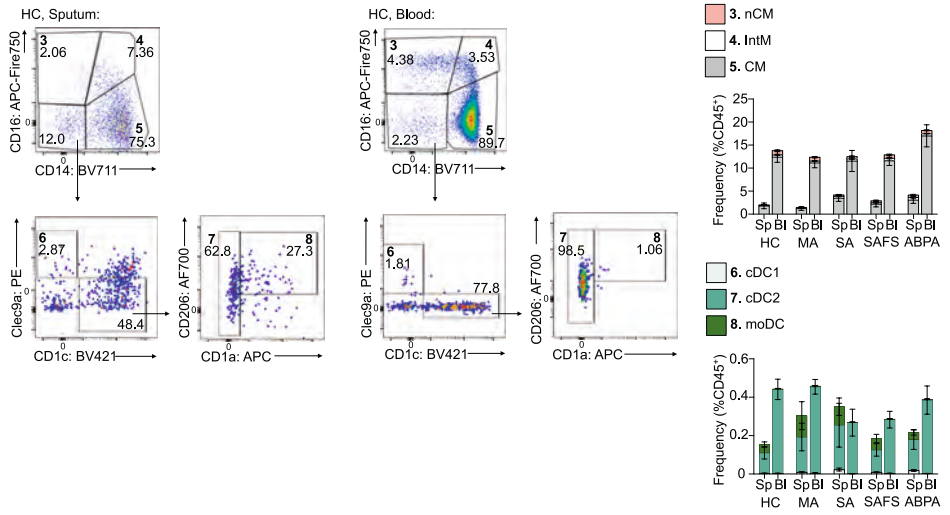
To highlight the cellular parameters which were most important for grouping patients, we extracted Gini coefficients from our RF model. These values indicate the relative importance of each parameter for classifying patients, with higher values indicating greater relative importance.[26] Of the top 20 parameters with the highest Gini

A

	Number of patients				
	HC	MA	SA	SAFS	ABPA
Sputum (airway)	20	20	16	19	19
Blood (circulatory)	14	14	11	19	19







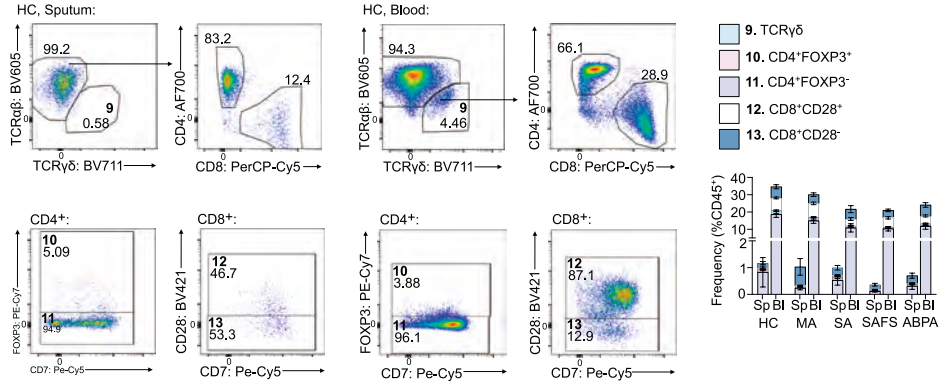


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Fig. 1. Identification of granulocyte, T cell and myeloid populations in the sputum and blood of severe asthma patients. A) Sputum and blood samples were collected from healthy controls (HC), mild asthmatics (MA), severe asthmatics (SA), severe asthmatics with fungal sensitisation (SAFS) and allergic bronchopulmonary aspergillosis (ABPA) patients. **B)** Frequency of granulocyte, T cell and myeloid populations in sputum (Sp) and blood (Bl), as percentage of $CD45^+$ cells. Error bars show standard error of the mean. **C)** Flow plots to show the gating of eosinophils (1, $CD66b^+Siglec8^+SSC^{hi}CD16^{lo}$) and neutrophils (2, $CD66b^+Siglec8^-SSC^{lo}CD16^{hi}$) in the sputum and blood of a HC. Stacked bar chart shows the frequency of eosinophils and neutrophils as percentage of $CD45^+$ cells. Error bars show standard error of the mean. **D)** Representative flow plots to show the gating of non-classical monocytes (nCM) (3, $CD11c^+HLA\text{-}DR^+CD14^-CD16^{hi}$), intermediate monocytes (IntM) (4, $CD11c^+HLA\text{-}DR^+CD14^{hi}CD16^{hi}$), classical monocytes (CM) (5, $CD11c^+HLA\text{-}DR^+CD14^{hi}CD16^-$), type 1 conventional dendritic cells (cDC1) (6, $CD11c^+HLA\text{-}DR^+CD14^-CD16^-CLEC9a^+$), type 2 conventional dendritic cells (cDC2) (7, $CD11c^+HLA\text{-}DR^+CD14^-CD16^-CD1c^+$) and monocyte derived dendritic cells (moDC) (8, $CD11c^+HLA\text{-}DR^+CD14^-CD16^-CD1c^+CD1a^+$) in the sputum and blood of a HC. Stacked bar charts show the frequency of these monocyte and DC subsets as percentage of $CD45^+$ cells. Error bars show standard error of the mean. **E)** Flow plots to show the gating of γδ T cells (9, $CD3^+TCR\gamma\delta^+$), $CD4^+$ T cell populations (10, $CD3^+CD4^+FOXP3^+$ and 11, $CD3^+CD4^+FOXP3^-$) and $CD8^+$ T cell populations (12, $CD3^+CD8^+CD28^+$ and 13, $CD3^+CD8^+CD28^-$) cells in the sputum and blood of a HC. Stacked bar chart shows the frequency of T cell subsets as percentage of $CD45^+$ cells. Error bars show standard error of the mean.

coefficients, 15 described the frequency of an immune cell population, whilst 5 described activation state (Fig. 2C). This suggests that cumulatively, the frequency of peripheral immune cell populations was more informative than activation state in deciphering asthma severity.

We then categorised the informative clustering parameters based on cell type and found that > 60 % related to T cells ($CD4^+$, $CD8^+$ and γδ T cells) (Fig. 2C). The expression of CD69 and HLA-DR on $CD4^+FOXP3^-$ T cells was highly informative for patient clustering, and was higher in severe disease groups (SA, SAFS, ABPA) than non-severe disease groups (Fig. 2D). As CD69 and HLA-DR are associated with T cell activation/antigen experience,[27–30] our data suggests an association between $CD4^+$ T cell activation and disease severity. This supports preclinical work which found $CD4^+$ T cells to be crucial for mediating allergic asthma.[24,31–32] Furthermore, in alignment with previous studies,[33–34] peripheral δγ cells and $CD8^+CD28^+$ T cells (CD28 being a co-stimulatory marker which is indicative of activation, maturity and antigen-experience)[35] were less frequent in severe disease patients than non-severe disease patients (Fig. 2D).

Overall, these data highlight that the frequency and activation of peripheral T cell populations are major identifiers of patients with severe airway disease, regardless of fungal sensitisation.

Sputum T cells, DCs and eosinophils provide robust identification of severe asthma patients

In contrast to the blood, we found that when Permutational MANOVA was performed on immune cell data derived from the sputum, significant differences were revealed between non-severe disease groups (HC and MA) and all severe disease groups (SA, SAFS, and ABPA) (Fig. 3A). However, SA, SAFS and ABPA patients could still not be separated from one another (Fig. 3A). These differences were also partially observed via PCA, as disease groups somewhat separated along PC1, accounting for 96.3 % of variance in the data (Supplementary Fig. 3B).

To assess which immune cell parameters from sputum samples were characteristic of severe asthma groups, we undertook RF analysis. In agreement with the Permutational MANOVA and PCA, MDS clustering separated non-severe and severe groups from one another, with an average error rate of 24 %, (Fig. 3B and Supplementary Fig. 4B). Of the 20 most informative parameters used to classify patients within the RF model (identified by Gini coefficient), 50 % related to immune cell population frequency and 60 % related to T cells (Fig. 3C). This was somewhat similar to findings in the blood (Fig. 2C). However, DC- and eosinophil-related parameters were also prominent in the sputum, and greater emphasis was placed on the activation state of immune cells than in the blood (Fig. 3C, Fig. 2C). This highlights the importance of considering both the frequency and activation of airway immune cells, to better understand the immunopathogenesis of severe airway disease.

Despite DCs playing a central role in driving allergy, few studies have characterised DC populations in the sputum of asthma patients.[36–37] Here, we found cDC2 HLA-DR expression was the most informative airway readout (Fig. 3C) and was notably elevated on cDC2s isolated from patients with severe disease compared to HC (Fig. 3D). This could be indicative of increased antigen presentation to T cells by DCs in severe disease, contributing to downstream inflammation.[38–40] Similarly, the expression of the C-type lectin receptor CD206, previously associated with mature cDC2 populations in the lung,[41] was elevated in the cDC2s of severe groups (Fig. 3D). cDC1s, another DC subset which may have a regulatory and homeostatic role in asthma,[42] were also informative and elevated in the sputum of patients with severe asthma (Fig. 3C & D).

The frequency of airway eosinophils and their expression of the activation and maturation markers CD69 and HLA-DR[43–45] were also significant in unbiased patient clustering (Fig. 3C & D). This was expected as eosinophilia is often correlated with SA[7–8,46] and is widely used in asthma diagnostics.[47] However, there was large variation in the proportion of sputum eosinophils within each disease group and this may be reflective of differences in treatment, co-morbidities and atopy status (Table 1).

Secreted factors in the sputum, particularly IFNγ, IL-9 and eotaxin, were indicative of asthma severity

Given that the cellular profile of the airway provided a strong indication of patient condition, we proceeded to quantify the concentration of 33 cytokines and secretory factors in sputum samples. Here pro-inflammatory, T2 and Type 17 (T17) cytokines/chemokines were measured, along with alarmins and growth factors. The majority of cytokines were elevated in individuals with severe disease than those with non-severe disease (Fig. 4A). Similarly to sputum eosinophilia, there was considerable variation in the concentration of secreted factors within groups (even in severe asthma patients). This could be due to differences in treatment regimens, co-morbidities or exacerbation status.[48–52]

Consistent with the cellular immune profile of the sputum, Permutational MANOVA revealed that cytokine profiles were significantly different when comparing severe (SA, SAFS, and ABPA) to non-severe (HC and MA) groups (Fig. 4B), and these clusters were confirmed via PCA (Supplementary Fig. 3C). However, it was still hard to distinguish between HC and MA patients, as well as SA, SAFS and ABPA (Fig. 4B). RF analysis of secreted factors also classified patients into non-severe and severe disease with a high degree of accuracy (Supplementary Fig. 4C), and patients clearly clustered into these two categories based on MDS analysis (Fig. 4C). Based on Gini coefficients extracted from the RF model, IFNγ was the most strongly weighted parameter in classifying patients and was significantly increased in the airways of SA, SAFS and ABPA patients compared to HC (Fig. 4D). This pro-inflammatory cytokine has consistently found to be raised in the airways of asthmatics during viral exacerbation[50–52] and has also been associated with neutrophilic asthma.[53] The T17 cytokines IL-8 and IL-17 were also informative and elevated in severe disease (Fig. 4D).

Thymic stromal lymphopoietin (TSLP) and IL-33 (shown to be important drivers of asthma) also guided the RF patient classification (Fig. 4D). However, in contrast to previous studies, TSLP and IL-33 were less concentrated in severe disease than non-severe disease[54–55] (Fig. 4F). Further, none of the T2 cytokines commonly used as biomarkers for eosinophilic asthma (IL-4, IL-5, IL-13) were ranked amongst

Table 1

Patient cohort demographics and clinical characteristics. Abbreviations: SAFS, severe asthma with fungal sensitisation; ABPA, allergic bronchopulmonary aspergillosis; BMI, body mass index; SD, standard deviation; COPD, chronic obstructive pulmonary disease; T2DM, Type 2 diabetes mellitus; TB, tuberculosis; *P. aeruginosa*, *Pseudomonas aeruginosa*; SABA, short-acting β 2-agonist; LABA, long-acting β 2-agonist; LAMA, long-acting muscarinic antagonists; LTRA, leukotriene receptor antagonist; ICS, inhaled corticosteroid; BDP, Beclometasone dipropionate equivalent; OCS, oral corticosteroid; IM, intramuscular; TDS, thrice daily; ppb; parts per billion.

Patient characteristic	Total cohort (n = 100)	Healthy controls (n = 20)	Mild Asthma (n = 20)	Severe Asthma (n = 20)	SAFS (n = 20)	ABPA (n = 20)
Age (median(range))	52 (19–78)	34.5 (19–63)	32.5 (19–70)	55 (36–75)	56.5 (28–76)	64.5 (48–78)
Sex (count (%))						
Male	33 (33)	5 (25)	4 (20)	4 (20)	10 (50)	10 (50)
Female	52 (52)	9 (45)	7 (35)	16 (80)	10 (50)	10 (50)
Not recorded	15 (15)	6 (30)	9 (45)	0	0	0
Smoking history (count (%))						
Never smoker	57 (57)	12 (60)	11 (55)	12 (60)	10 (50)	12 (60)
Ex-smoker	28 (28)	4 (20)	6 (30)	4 (20)	7 (35)	7 (35)
Current smoker	10 (10)	4 (20)	1 (5)	2 (10)	2 (10)	1 (5)
Not recorded	5 (5)	0	2 (10)	2 (10)	1 (5)	0
Pack year history (median (range))	8.5 (0.5–123)	5 (0.5–21)	5 (1–14)	16.5 (1–44)	6.5 (1–33)	12.5 (5–123)
Ethnicity (count (%))						
White − British	65 (65)	11 (55)	1 (5)	19 (95)	18 (90)	17 (85)
White − Other	4 (4)	1 (5)	1 (5)	0	0	1 (5)
Asian British	1 (1)	0	0	0	1 (5)	0
Black British	1 (1)	0	1 (5)	0	0	0
Not recorded	29 (29)	8 (40)	17 (85)	1 (5)	1 (5)	2 (10)
BMI (kg/m^2 (mean ± SD)	29.1 ± 7.7	35.1 ± 0	24.6 ± 2.7	32.4 ± 10.3	28.4 ± 6.8	27.1 ± 5.4
Atopy (count (%))						
Allergic rhinitis	23 (23)	0	10 (50)	4 (20)	7 (35)	2 (10)
Atopic dermatitis	8 (8)	0	3 (15)	2 (10)	3 (15)	0
Nasal polyposis	4 (4)	0	0	2 (10)	1 (5)	1 (5)
Co-morbidities (count (%))						
COPD	3 (3)	0	2 (10)	1 (5)	0	0
Bronchiectasis	17 (17)	0	0	5 (25)	5 (25)	7 (35)
T2DM	3 (3)	0	1 (5)	1 (5)	1 (5)	0
Previous TB	2 (2)	0	0	0	1 (5)	1 (5)
Vocal cord dysfunction	1 (1)	0	0	1 (5)	0	0
P. aeruginosa colonisation	1 (1)	0	0	0	0	1 (5)
Treatments not recorded (count (%))	6 (6)	0	2 (10)	1 (5)	2 (10)	1 (5)
Asthma treatment (count (%))						
SABA	71 (76)	0	16 (89)	18 (95)	18 (100)	19 (100)
LABA monotherapy	6 (6)	0	0	3 (16)	2 (11)	1 (5)
LAMA monotherapy	17 (18)	0	0	3 (16)	9 (50)	5 (26)
LTRA	24 (26)	0	1 (6)	7 (37)	10 (56)	6 (32)
Theophylline	14 (15)	0	2 (11)	5 (26)	3 (17)	4 (21)
Mucolytics	16 (17)	0	0	7 (37)	4 (22)	5 (26)
Nebulisers	9 (10)	0	0	7 (37)	2 (11)	0
ICS monotherapy	11 (12)	0	9 (50)	1 (6)	0	1 (5)
ICS/LABA	51 (54)	0	5 (28)	17 (89)	14 (78)	15 (79)
ICS/LABA/LAMA	0	0	0	0	0	0
BDP equivalent dose in mcg (count (%))						
500	7 (7)	N/A	4 (20)	1 (5)	1 (6)	1 (5)
1000	45 (48)		9 (50)	15 (79)	10 (56)	11 (58)
2000	9 (10)		2 (11)	1 (5)	3 (17)	3 (16)
Steroid use (count (%))						
Therapeutic OCS (*30–40 mg*)	3 (3)	0	0	1 (5)	1 (6)	1 (5)
Maintenance OCS	23 (24)	0	0	11 (55)	6 (33)	6 (32)
Triamcinolone IM	7 (7)	0	0	5 (25)	2 (10)	0
Adrenal insufficiency dosing (*Hydrocortisone TDS*)	6 (6)	0	1 (5)	1 (5)	3 (17)	1 (5)
Biologic use (count (%))						
Omalizumab	9 (10)	0	0	2 (11)	6 (33)	1 (5)
Prior use	0	0	0	0	0	0
Antibiotic use (count (%))						
Azithromycin	18 (19)	0	0	9 (47)	5 (28)	4 (21)
Other (drug name)	5 (5)	0	0	1 (5) (Doxycycline)	3 (17) (Colomycin, Tobramycin)	1(5) (Colomycin)
Antifungal use (count (%))						
Itraconazole	19 (20)	0	0	1 (5)	0	11 (58)
Voriconazole	1 (1)	0	0	0	1 (6)	1 (5)
Ambisome	1 (1)	0	0	0	3 (17)	0
Amphotericin	5 (5)	0	0	0	3 (17)	2 (11)
Previous use	3 (3)	0	0	0	1 (6)	0
Immunosuppression (count (%))	6 (6)	0	0	4 (21)		1 (5)
Lung function						
Not recorded (count (%))	37 (37)	19 (95)	10 (50)	3 (15)	0	5 (25)

(*continued on next page*)

Table 1 (*continued*)

Patient characteristic	Total cohort (n = 100)	Healthy controls (n = 20)	Mild Asthma (n = 20)	Severe Asthma (n = 20)	SAFS (n = 20)	ABPA (n = 20)
Incomplete data (count (%))	35 (35)	1 (5)	8 (40)	8 (40)	9 (45)	9 (45)
FEV1 % predicted (mean ± SD)		115 ± 0	105 ± 12	74 ± 22	71 ± 22	73 ± 22
FVC % predicted (mean ± SD)		116 ± 0	89 ± 36	96 ± 19	91 ± 17	100 ± 22
FEV1/FVC ratio (mean ± SD)		0.85 ± 0	0.84 ± 0.03	0.65 ± 0.12	0.63 ± 0.14	0.58 ± 0.13
Residual volume % predicted (mean ± SD)	(Recorded in 3 patients only)	N/A	N/A	92 ± 57	119 ± 46	89 ± 18
DLCO % predicted (mean ± SD)		83 ± 0	N/A	102 ± 14	87 ± 15	94 ± 14
FeNO in ppb (median (range))		N/A	11.5 (10–13)	N/A	N/A	58 (0)

the top 20 most informative clustering parameters (Fig. 4D). Nevertheless, they were still significantly elevated in severe disease groups compared to HC (Supplementary Fig. 5).

Taken together, these data highlight an association between IFNγ, IL-9 and eotaxin and asthma severity, regardless of fungal sensitisation. No difference between SA, SAFS and ABPA were detected, so higher resolution techniques may be required to detect subtle differences between these groups.

Discussion

We collected paired blood and sputum samples from a large asthma cohort to define immune signatures that associate with SAFS and ABPA. Unbiased machine learning analysis of our datasets revealed the immune profile of HC and MA patients to be distinct from patients with SA, SAFS and ABPA. Furthermore, sputum samples, reflecting the airway, were more effective than blood samples for discriminating between these patient groups.

Due to difficulties associated with obtaining patient airway samples, most asthma research has previously been conducted on blood samples.[56] Thus, many peripheral immune cell populations, including eosinophils, neutrophils, T cells, monocytes and DCs, have been shown to differ between healthy individuals and those with severe asthma.[32] However, understanding which of these populations are most significant in driving disease is complex and likely to be driven by diverse contributory factors. Here, our RF analysis showed peripheral T cells to be the prominent population that distinguished between non-severe and severe airway disease, particularly $CD4^+$ T cell activation and the frequency of TCRγδ and $CD8^+CD28^+$ populations (Fig. 2C & D). This supports previous studies which have associated these factors with asthma.[33–34,57] Further, in the sputum, the expression of CD69 and HLA-DR on airway-associated $CD4^+FOXP3^-$ T cells was important in guiding clustering analysis (Fig. 3C). We suggest that these cells could be the source of the elevated IL-13 and IL-17 concentrations seen in the sputum of severe disease groups (Fig. 4A & D, Supplementary Fig. 5). This would align with findings from our murine model of fungal allergic airway inflammation, which showed that IL-13^+ and IL-17^+ $CD4^+$ T cells drive airway eosinophilia and neutrophilia.[24] Our data identifies the importance of both systemic and local T cell activation in severe asthma, regardless of fungal sensitisation.

Further analysis of sputum samples revealed that in addition to T cells, DC subsets (cDC1s and cDC2s) could discriminate between HC/MA and SA/SAFS/ABPA patients (Fig. 3C & D). Whilst the role of cDC1s in asthma is debated,[42] previous studies in mouse models have shown that cDC2s are critical mediators of allergic inflammation to fungal and non-fungal allergens.[24] In humans, a recent study on mild asthmatic patients revealed that airway cDC2s were key responders to allergen challenge[58] and here, we observed that their activation status helped to define patient condition (Fig. 3C & D). To our knowledge, this study is the first to highlight the importance of cDC2 activation in the airways of SAFS and ABPA patients. However, future work is needed to understand the functional capacity of cDC1s vs cDC2s and whether the elevated expression of mediators such as HLA-DR could be driving T cell activation in severe asthma.

In comparison to DCs, eosinophils are well characterised in human asthma and are used as a core diagnostic measure of T2 inflammation.[47] In support of their clinical use, we found that sputum eosinophil frequency was the fourth most important airway factor in identifying patients with severe disease (Fig. 3C) and tended to be elevated in SA than HC (Fig. 3D). Comparatively, blood eosinophils were less informative in guiding RF analysis as their frequency was only ranked 11th of the top 20 influential peripheral factors (Fig. 2C). However, this could be explained by the fact that all patients within our cohort were receiving treatment for their condition (Table 1). 30 % of patients were on corticosteroids (either therapeutically at 30–40 mg, or as maintenance at ≤ 10 mg) and 10 % were receiving Omalizumab, both of which are known to reduce peripheral eosinophil counts and control T2 inflammation.[59–62] If samples had been collected prior to treatment, it is likely that eosinophilia would have been more pronounced in severe disease groups. The impact of recently-approved biologic therapies on the immune environment of these patients remains an important question to address.

As seen with the flow cytometry data, when RF analysis was applied to secreted factors from sputum supernatants, patients clustered into non-severe and severe disease groups (Fig. 4C). Out of the 34 secreted factors assessed, IFNγ, IL-9 and eotaxin were most important in predicting severe disease (Fig. 4D). These factors have been previously associated with asthma, specifically IFNγ in the context of viral exacerbation[50–52] and eotaxin and IL-9 in the context of T2 inflammation and allergy.[63–67] These cytokines could therefore complement existing diagnostic strategies and have the potential to stratify patients into subgroups (e.g. T2H/T2L). This should be explored in a larger cohort study, along with the cellular source of these cytokines.

In summary, our work demonstrates how RF can be used to identify key immune factors which distinguish health and disease. We found that the immune profile of the sputum was a more effective indicator of severe airway disease than that of the blood, highlighting the value of obtaining patient airway samples for basic research. Our data has highlighted a key association between sputum T cells, cDC2s and eosinophils with severe airway disease, and invites future studies to investigate the role of these populations on a mechanistic level. However, regardless of sample type, there were no striking differences in the immune environment of SA, SAFS and ABPA patients. To identify the mechanisms which underpin fungal sensitisation in severe asthma, more sensitive techniques such as single cell RNA-sequencing or *ex vivo* cell stimulation with fungal antigens may be required. This would improve understanding of disease pathology and potentially guide the development of improved diagnostic and treatment strategies for SAFS and ABPA.

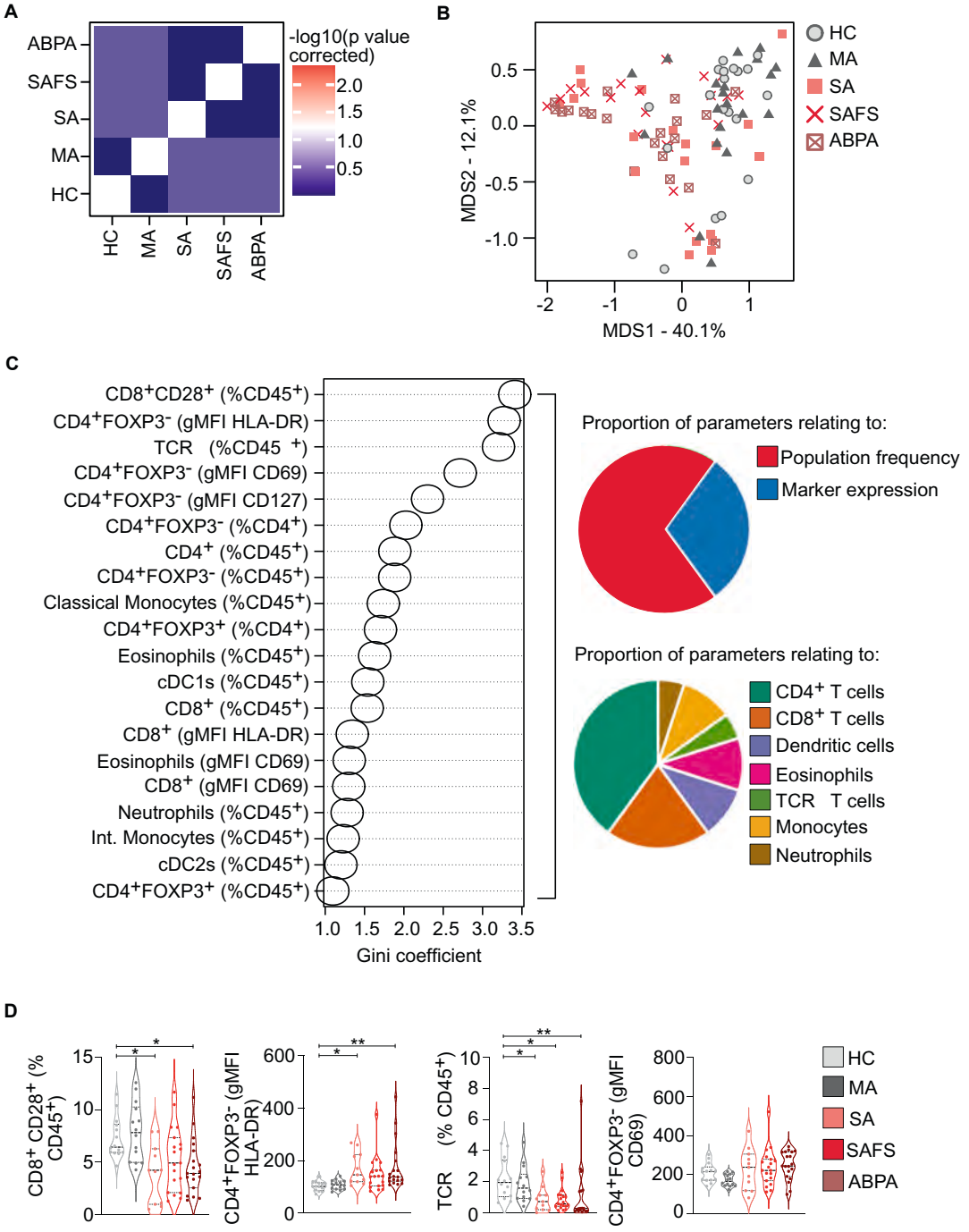

Fig. 2. T cell abundance and activation in peripheral blood identifies severe asthma patients. Multivariate statistical analysis was performed on cellular immune parameters quantified by flow cytometry on cells isolated from peripheral blood. **A)** Tile plot representing the results of pairwise multivariate statistical comparisons between groups via Permutational MANOVA. **B-D)** Random forests (RF) analysis was performed using the randomForests package in R. **B)** Classical multi-directional scaling ordination plot calculated from sample proximity within the RF model. Each data point represents a patient, and symbols are indicative of disease group. **C)** The top 20 most informative parameters for guiding patient clustering, ranked via Gini coefficient. Pie charts to show the categorisation of these parameters as either 1) relating to immune cell population frequency or activation status, or 2) cell type. **D)** Violin plots show the top four most informative clustering parameters. Each dot represents an individual patient, and quartiles are shown by horizontal lines. $p < 0.05$ (*), $p < 0.01$ (**), compared to HC.

Material and methods

Participant selection

Patients for this study were recruited from specialist clinics at the National Aspergillosis Centre in Manchester between January and June 2018. Paired blood and sputum samples were obtained alongside demographic data and clinical information. Patients were recruited to each of the following cohorts: healthy controls (HC), mild asthmatics (MA), severe asthmatics (SA), severe asthmatics with fungal sensitisation (SAFS), and allergic bronchopulmonary aspergillosis (ABPA) patients. Power calculations indicated significant differences between groups would be detected if 15 patients were recruited per group (assuming $p < 0.05$, 80 % power, with an assumed standard deviation of 1.5). HCs were defined as adults aged ≥ 18 with no significant past medical history or current prescription drug use. MAs were defined as those with objective

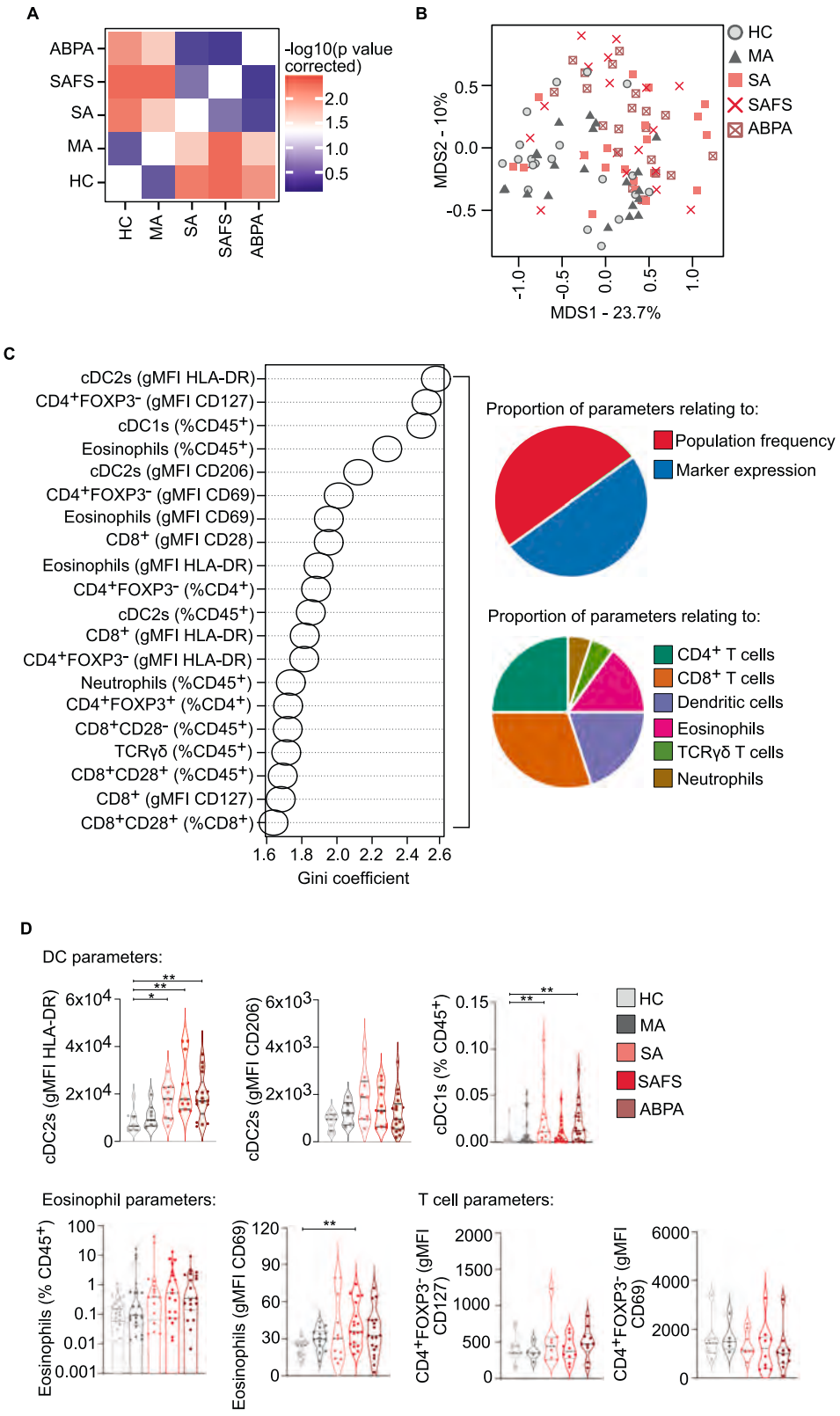

Fig. 3. Abundance and activation of sputum T cells, dendritic cells and eosinophils identifies severe asthma patients. Multivariate statistical analysis was performed on cellular immune parameters quantified by flow cytometry on cells isolated from sputum. **A)** Tile plot representing the results of pairwise multivariate statistical comparisons between groups via Permutational MANOVA. **B-D)** Random forests (RF) analysis was performed using the randomForests package in R. **B)** Classical multi-directional scaling ordination plot calculated from sample proximity within the RF model. Each data point represents a patient, and symbols are indicative of disease group. **C)** The top 20 most informative parameters for guiding patient clustering, ranked via Gini coefficient. Pie charts to show the categorisation of these parameters as either 1) relating to immune cell population frequency or activation status, or 2) cell type. **D)** Violin plots show the top four most informative clustering parameters. Each dot represents an individual patient, and quartiles are shown by horizontal lines. $p < 0.05$ (*), $p < 0.01$ (**), compared to HC.

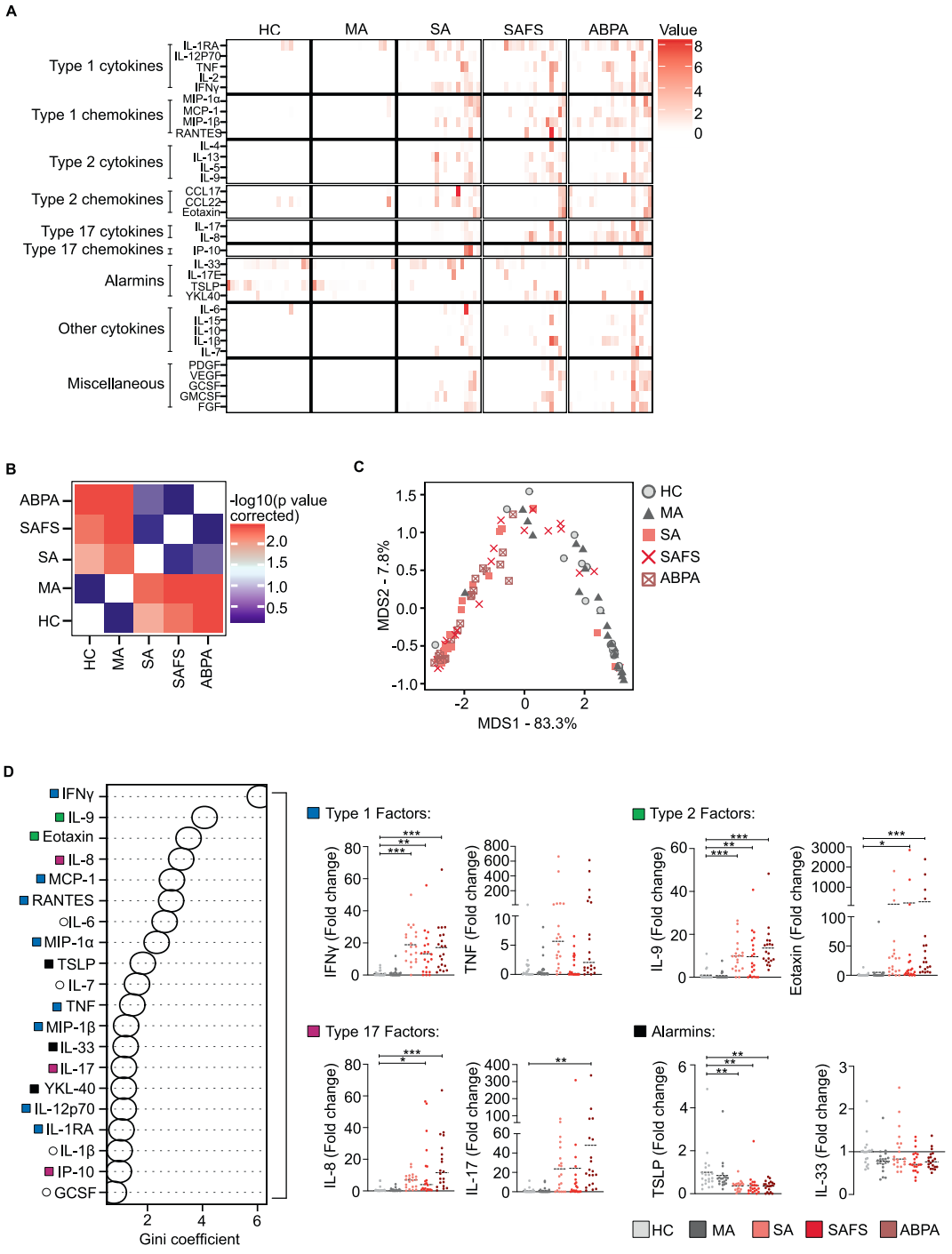

Fig. 4. A range of secreted factors in the sputum predict asthma severity in a Random Forest analysis. (A) Heat map to show the concentration of secreted factors in each patient sputum sample, as measured by Luminex and ELISAs. **(B-D)** Multivariate statistical analysis performed on these secreted factors. **(B)** Tile plot representing the results of pairwise multivariate statistical comparisons between groups via Permutational MANOVA. **(C-D)** Random forests (RF) analysis was performed using the randomForests package in R. **(C)** Classical multi-directional scaling ordination plot calculated from sample proximity within the RF model. Each data point represents a patient, and symbols are indicative of disease group. **(D)** The top 20 most informative secreted factors for guiding patient clustering, ranked via Gini coefficient. Blue squares indicate Type 1 factors, green squares indicate Type 2 factors, purple squares indicate Type 17 factors, black squares indicate alarmins and white circles indicate 'other'. Column charts show the most informative Type 1 factors (IFNγ, TNFα), Type 2 factors (IL-9, eotaxin), Type 17 factors (IL-8, IL-17) and alarmins (TSLP, IL-33), plotted as fold change, relative to HC mean. Each point represents an individual patient, and dotted lines show the mean for each patient group. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

evidence and a formal clinical diagnosis of asthma with persistent symptoms and use of reliever inhalers > 2 days/week, but not daily. SAs were defined in keeping with the European Respiratory Society/American Thoracic Society definition of patients having uncontrolled disease despite use of high dose ICS and ≥ 1 additional controller medications.[6] SAFS was defined as previously described and the presence of ABPA excluded.[6] ABPA was defined as per International Society for Human & Animal Mycology (ISHAM) criteria at the time of recruitment (2018). All

participants gave informed written consent. We acknowledge the Manchester Allergy, Respiratory and Thoracic Surgery Biobank for supporting this project and thank the study participants for their contribution, Rec: 20/NW/0302, IRAS: 285,126 (North West − Haydock Research Ethics Committee).

Sample collection and cell isolation

Sputum samples (spontaneous in SA, SAFS and ABPA patients or induced by hypertonic saline in HC and MA patients) were collected and processed as previously described[68]. Samples were centrifuged and supernatants and cells cryopreserved at −150 °C, with cells resuspended in freezing media (50 % DMEM, 10 % DMSO and 50 % FBS (all Sigma) controlled freezing of sputum cells was obtained by use of a CoolCell Freezer container (Corning)).

Blood samples, taken at same time as sputum collection were collected in EDTA-coated tubes and transported to the laboratory at room temperature. Cells were resuspended in sterile water for 10 s, to lyse red blood cells and then cryopreserved in freezing media and stored at −150 °C. Controlled freezing of cells isolated from sputum and blood was obtained by use of a Mr Frosty freezer container (Nalgene).

Flow cytometry

Sputum and blood samples were thawed in a 37 °C water bath and resuspended in pre-warmed RPMI + 10 % FCS + DNase. Following centrifugation, the pellet was re-suspended in ice cold FACS buffer (PBS containing 2 % FBS and 2 mM EDTA). Cells were washed with PBS and stained for viability with Live/Dead Blue™ (1:2000; ThermoFisher). Cells were then blocked with Human FcBlock (BioLegend) in FACS buffer, before being stained for surface markers at 4 °C for 30 min. Surface stain was then removed by washing the samples twice in FACS buffer. To fix, cells were then before resuspended in 1 % paraformaldehyde (PFA) in PBS and incubated at room temperature for 10 min. PFA was removed and cells were washed three times in eBioscience permeabilization buffer (ThermoFisher) for the staining of intracellular cytokines at 4 °C for 60 min. Antibody details can be found in Supplementary Table 1. Data was acquired on BD Fortessa and analysis was undertaken using FlowJo™ v10.0 Software (BD Life Sciences). Sentinel gating was used to maximise the number of populations that we could identify from our 16-colour panel. Briefly, markers which were not co-expressed by the same immune cell population were assigned the same fluorophore. For example, the T cell marker CD8 and the B cell marker CD19 were both assigned BB700. Other markers which shared the same fluorophore were TCRγδ and CD14, Siglec-8 and TCRab, FOXP3 and CD1a, CD4 and CD206, Clec9a and CD127. Gating strategies and representative flow cytometry plots are shown in Supplementary Fig. 1. Due to the high variation in sputum sample quality and cell count, immune cell populations were excluded from downstream analysis if event number was below the set threshold value (Supplementary Table 4).

Luminex and ELISAs

To quantify the concentration of various soluble factors in sputum supernatants, a Bio-Plex Pro Human Cytokine 27-plex Assay (BioRad) was performed per manufacturers instructions. R&D DuoSet ELISAs kits were used to quantify the concentration of CCL17, CCL22, TSLP, IL-33, YKL40 and IL-17E according to the manufacturers instructions. Where concentration was below the limit of detection, a substitute value of $1x10^{-7}$ was used to enable subsequent fold change calculations.

Statistical analysis

Statistical analysis was performed using Prism v9 (GraphPad) and R (version 4.4.1). Normality was tested for using the Shapiro-Wilk test. Disease groups were then compared to healthy controls using a parametric one-way ANOVA Dunnett's multiple comparisons test or a non-parametric Kruskal-Wallis Dunn's multiple comparison test, with a 95 % confidence interval. p values are represented as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Multivariate testing using Permutational MANOVA was performed using the 'adonis.pair' function from the 'EcolUtils' package (version 0.1) with p values corrected for the false discovery rate (fdr). Principle components analysis was performed using the 'prcomp' function.

Random forest analysis

Random forests analysis was performed in R (version 4.4.1) using the 'randomForest' package (version 4.7–1.1). Prior to analysis, missing data points were imputed using the 'rfImpute' function. For each model the number of trees was set to 1000 and mtry was set to the level which produced the lowest out of bag error. Dimensionality reduction was performed using classical multidimensional scaling (MDS) by generating a distance matrix from proximity valuescalculated by the Random forest model using the 'dist' function then running 'cmdscale'.

CRediT authorship contribution statement

Emily L. Plumpton: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Visualization. **Stefano A.P. Colombo:** Visualization, Formal analysis, Data curation, Writing – review & editing. **Matthew Steward:** Writing – original draft, Formal analysis, Data curation. **Sheila L. Brown:** Methodology, Investigation. **Saba Khan:** Methodology, Investigation. **Gaël Tavernier:** Methodology, Investigation. **Helen Francis:** Investigation. **Hazel Platt:** Methodology, Investigation. **Tracy Hussell:** Methodology, Investigation. **William Horsnell:** Writing – review & editing, Writing – original draft. **David Denning:** Investigation. **Robert Niven:** Investigation. **Angela Simpson:** Methodology, Investigation. **Andrew S. MacDonald:** Investigation, Conceptualization. **Peter C. Cook:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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submission.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mucimm.2025.11.004.

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