IgA$^+$ memory B-cells are significantly increased in patients with asthma and small airway dysfunction

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Shareable abstract (@ERSpublications)
Circulating B-cells are altered in asthma patients. In particular, IgA$^+$ memory B-cells are significantly increased in patients with impaired lung function, especially of the small airways, suggesting a contribution to inflammation in the peripheral lung. https://bit.ly/3r0xBNA

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Abstract

Background Comprehensive studies investigated the role of T-cells in asthma which led to personalised treatment options targeting severe eosinophilic asthma. However, little is known about the contribution of B-cells to this chronic inflammatory disease. In this study we investigated the contribution of various B-cell populations to specific clinical features in asthma.

Methods In the All Age Asthma Cohort (ALLIANCE), a subgroup of 154 adult asthma patients and 28 healthy controls were included for B-cell characterisation by flow cytometry. Questionnaires, lung function measurements, blood differential counts and allergy testing of participants were analysed together with comprehensive data on B-cells using association studies and multivariate linear models.

Results Patients with severe asthma showed decreased immature B-cell populations while memory B-cells were significantly increased compared with both mild–moderate asthma patients and healthy controls. Furthermore, increased frequencies of IgA$^+$ memory B-cells were associated with impaired lung function and specifically with parameters indicative for augmented resistance in the peripheral airways. Accordingly, asthma patients with small airway dysfunction (SAD) defined by impulse oscillometry showed increased frequencies of IgA$^+$ memory B-cells, particularly in patients with mild–moderate asthma. Additionally, IgA$^+$ memory B-cells significantly correlated with clinical features of SAD such as exacerbations.

Conclusions With this study we demonstrate for the first time a significant association of increased IgA$^+$ memory B-cells with asthma and SAD, pointing towards future options for B-cell-directed strategies in preventing and treating asthma.

Introduction

Asthma is one of the most prevalent chronic respiratory diseases characterised by airway inflammation, airway hyperreactivity and impaired lung function with obstruction of the central and peripheral airways [1, 2]. In the past decades, a better understanding of distinct phenotypes and endotypes of this heterogeneous disease supported the development of personalised therapeutic approaches, mainly directed against type 2 cytokines in severe eosinophilic asthma [3]. In contrast, knowledge of the impact of Bcells on asthma is still very limited and mostly acknowledges their role in allergic asthma as IgE producers [4]. More recently, research revealed immunomodulatory functions of regulatory B-cells on allergic airway inflammation [5] and allergen tolerance [6]. Additionally, we could show that B-cells control airway hyperreactivity and airway remodelling in a murine asthma model [7], pointing towards a possible role of B-cells for future diagnostic and preventive strategies in asthma.

The peripheral B-cell compartment consists of various populations ranging from immature so-called transitional B-cells to mature naïve B-cells. Activation of naïve B-cells leads to highly specialised antigen-experienced $CD27^+$ memory B-cells or plasma cells producing IgM, IgA, IgG or IgE [8, 9]. Additionally, less antigen-specific and therefore polyreactive IgA is produced by $CD27^-$ memory B-cells which play a role in mucosal host–microbiome homeostasis [10]. Memory B-cells recirculate in blood, secondary lymphoid tissues [11] and mucosal organs such as the lung [12], and their re-activation results in a strengthened immunoglobulin response [11, 13].

In particular, IgA and IgA^+ B-cells are crucial for pulmonary mucosal immune defence [14] and also show immunomodulatory properties [15]. Histology studies in chronic obstructive pulmonary disease (COPD) connected IgA^+ B-cells and locally impaired secretion of IgA to inflammation of the small airways [16, 17]. This is of particular interest as inflammation and obstruction of the peripheral airways (bronchioles <2 mm) is also an important clinical feature of asthma called small airway dysfunction (SAD) [18, 19]. SAD occurs in patients with mild–moderate and severe asthma, and significantly affects exacerbation rates, quality of life and daily physical activity [20, 21]. While lung function and imaging correlates of SAD have been frequently investigated in recent years [18, 21], little is known about the inflammatory processes contributing to SAD due to the relative inaccessibility of the small airways for cellular specimen collection.

Based on our previous findings in experimental asthma mouse models [5, 7], we hypothesised that B-cells influence asthma pathogenesis in humans and are linked to specific clinical characteristics in asthma patients. We therefore analysed immature, mature and memory B-cells in peripheral blood of asthma patients and healthy controls of the All Age Asthma Cohort (ALLIANCE). We used supervised and unsupervised statistical methods to search for associations between specific B-cell populations and essential clinical asthma features such as disease severity, markers of airway inflammation and lung function. Overall, we aimed to delineate the influence of B-cells on inflammatory processes driving asthma pathogenesis or specific traits to address the existing knowledge gap about B-cells and asthma, and to explore the potential of B-cells for disease phenotyping and diagnostics to improve personalised asthma care.

Materials and methods

Subjects and sample collection

B-cell analysis was done in probands with available blood specimens comprising 154 adult patients and 28 healthy controls of the ALLIANCE cohort, a longitudinal multicentre clinical cohort of the German Center for Lung Research (DZL) recruiting children with pre-school wheeze and asthma as well as adult asthma patients [22]. All local medical ethics committees of the participating centres approved the study protocol and all participants gave their written informed consent. Adults were recruited at the DZL sites of the Airway Research Center North (ARCN). The study was registered at ClinicalTrials.gov (adult arm: NCT02419274). Study design, inclusion and exclusion criteria, detailed data, and biomaterial collected at yearly study visits have been reported elsewhere [22]. Adult patients with asthma diagnosed according to international [23] and national guidelines [24] were eligible for inclusion as well as healthy controls without a previous asthma diagnosis and respective clinical symptoms. Further information concerning study design, methods and definition of clinical variables is available in the supplementary material.

B-cell characterisation

Isolated peripheral blood mononuclear cells were used for phenotypic characterisation of B-cell subpopulations. Cells were blocked, stained and analysed *via* flow cytometry. Further details are specified in the supplementary material.

Statistical analysis

The analysis was done using R version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria) with the R packages stats (version 4.0.4), q-value (version 2.20.0) and ggpubr (version 0.4.0).

For patient characterisation, the median (interquartile range) or percentage was calculated for continuous or categorical variables, respectively. The Wilcoxon test or Chi-squared test of independence was used to calculate p-values.

The association between pairs of B-cell populations and clinical variables was analysed using the Kruskal–Wallis test for categorical and Spearman's correlation for continuous clinical variables. To adjust for multiple testing, the Benjamini–Hochberg procedure was used. For continuous variables, linear regression lines were generated and for categorical variables the p-values (using the Wilcoxon test) between the categories were calculated. The same method was also used to examine the association between pairs of clinical variables.

Multivariate linear regression was used to assess the relationship between B-cell populations (percentage of $CD19^+$ B-cells) and all clinical variables as used in the association analysis while accounting for additional confounders such as age and oral corticosteroid (OCS) intake. To determine the significance of the clinical variable term, a model comparison approach was taken. A null model consisting of age and OCS (but without the clinical variable of interest) was compared with the full model consisting of the clinical variable, age and OCS using ANOVA. The resulting ANOVA derived p-values were subsequently corrected for multiple testing using Storey q-values [25].

To define SAD, the upper limit of normal and percentage predicted values of impulse oscillometry (IOS) parameters were determined according to the 95th centile of a German cohort of healthy adults [26].

To analyse the relationship between SAD and IgA^+ B-cells and clinical variables, a multivariate linear model was built. Features for the model were chosen from age, gender, exhaled nitric oxide fraction (F_{ENO}), sputum and blood eosinophils, sum of allergen-specific IgE, smoking (pack-years), body mass index (BMI), and OCS intake using a stepwise model selection by Akaike Information Criteria. Further information regarding the clinical variables is available in the supplementary material.

Results

Study population

The study population included 154 patients with asthma and 28 healthy subjects from the ALLIANCE cohort. Mean age was comparable between patients and controls. 40% of patients suffered from severe asthma according to the European Respiratory Society/American Thoracic Society guidelines [27]. More details are presented in tables 1 and 2.

Patients with severe asthma have altered frequencies of B-cell populations

We investigated peripheral B-cells of patients and healthy volunteers by flow cytometry (supplementary figure S1).

Percentages of different B-cell subpopulations were significantly associated with important clinical characteristics such as asthma severity, exacerbation frequency, blood neutrophils, sputum eosinophilia and lung function parameters (figure 1 and supplementary table S3).

Patients with severe asthma showed a significant reduction of the immature early transitional 1 (T1) and late transitional 2 (T2) B-cell populations compared with patients with mild–moderate asthma and healthy subjects. Similarly, percentages of mature naïve B-cells were diminished in patients with severe compared with mild–moderate asthma, but comparable to the percentage of healthy volunteers (figure 2a, supplementary figure S2a and supplementary table S3). Conversely, proportions of unswitched $CD27^+IgM^+$ as well as class-switched $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B-cells were strongly increased in severe compared with mild–moderate asthma. In addition, $CD27^+IgM^+$ and $CD27^+IgA^+$ but not $CD27^+IgG^+$ memory B-cells were increased in severe asthma patients compared with healthy controls (figure 2a and supplementary figure S2a).

Patients with regular OCS intake showed similar findings as patients with severe asthma (figure 2b and supplementary figure S2b). An increased frequency of $CD27^+IgA^+$ memory B-cells occurred in uncontrolled disease according to the 2019 Global Initiative for Asthma definition [23] and was also

TABLE 1 Clinical characteristics of patients with asthma and healthy controls

	Asthma patients (n=154)	Healthy controls (n=28)	p-value
Age (years)	53.1 (45.0–64.9)	56.2 (36.1–68.7)	0.97
BMI (kg·m^{-2})	27.2 (24.4–30.7)	24.9 (22.4–27.1)	0.012
Female	86 (56)	12 (43)	0.288
Current or ex-smoker ⩾10 pack-years	40 (26)	4 (14)	0.276
Atopy, blood and sputum differential counts			
Atopy	88 (59)	9 (33)	0.024
Blood eosinophil granulocytes (×10^3 µL^{-1})	0.29 (0.14–0.49)	0.12 (0.07–0.17)	<0.001
Blood neutrophil granulocytes (×10^3 µL^{-1})	4.32 (3.37–5.88)	3.20 (2.53–3.59)	<0.001
Sputum eosinophil granulocytes (%)	1.8 (0.5–6.7)	0.1 (0.0–0.5)	<0.001
Sputum neutrophil granulocytes (%)	56.0 (32.0–73.1)	53.4 (21.4–72.8)	0.490
Blood eosinophils ⩾300 µL^{-1}	75 (49)	2 (7)	<0.001
Sputum eosinophils ⩾2%	65 (49)	0 (0)	<0.001
Lung function			
FEV_1 (z-score)	−1.53 (−2.40– −0.49)	−0.03 (−0.49–0.46)	<0.001
FEV_1 (% pred)	78.55 (65.18–92.8)	99.62 (92.26–107.68)	<0.001
FEV_1/FVC (z-score)	−1.73 (−2.69– −0.81)	−0.65 (−0.95– −0.12)	<0.001
FEV_1/FVC (% pred)	84.95 (74.09–92.99)	94.52 (90.95–98.94)	<0.001
$FEF_{25–75\%}$ (z-score)	−1.69 (−2.78– −0.80)	−0.43 (−0.77–0.06)	<0.001
$FEF_{25–75\%}$ (% pred)	51.55 (30.23–75.27)	86.05 (73.52–101.97)	<0.001
F_{ENO} (ppb)	26.0 (16.0–44.0)	17.0 (13.0–19.8)	<0.001
R5−R20 (kPa·L^{-1}·s^{-1})	0.11 (0.06–0.19)	0.03 (0.01–0.06)	<0.001
R5−R20 (% pred)	186 (107–331)	93 (30–125)	<0.001
AX (kPa·L^{-1}·s^{-1})	0.67 (0.31–1.61)	0.17 (0.10–0.27)	<0.001
AX (% pred)	244 (116–498)	60 (25–107)	<0.001
FRES (Hz)	17.07 (12.68–21.29)	9.44 (8.45–13.08)	<0.001
FRES (% pred)	134 (109–166)	98 (80–124)	<0.001

Data are presented as median (interquartile range) or n (%), unless otherwise stated. BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; $FEF_{25–75\%}$: forced expiratory flow at 25–75% of FVC; F_{ENO}: exhaled nitric oxide fraction; R5–R20: resistance at 5 Hz−resistance at 20 Hz; AX: reactance area; FRES: resonance frequency.

TABLE 2 Clinical characteristics of asthma patients (n=154)

Disease duration (years)	19 (8–32)
Adult-onset asthma	102 (67)
Patients with ⩾1 severe exacerbations	82 (53)
Asthma severity	
Mild–moderate	92 (60)
Severe	62 (40)
Asthma Control Test score	20 (14–23)
GINA control status	
Controlled	48 (31)
Partly controlled	46 (30)
Uncontrolled	60 (39)
Medication	
Mean ICS dose[#] (µg·day^{-1})	450±480
LTRA	25 (16)
LABA	129 (84)
LAMA	37 (24)
OCS	36 (23)
Omalizumab	5 (3)
Mepolizumab	2 (1)

Data are presented as median (interquartile range), n (%) or mean±SD, unless otherwise stated. GINA: Global Initiative for Asthma; ICS: inhaled corticosteroids; LTRA: leukotriene antagonists; LABA: long-acting β_2-agonists; LAMA: long-acting muscarinic antagonists; OCS: oral corticosteroids. [#]: fluticasone-equivalent.

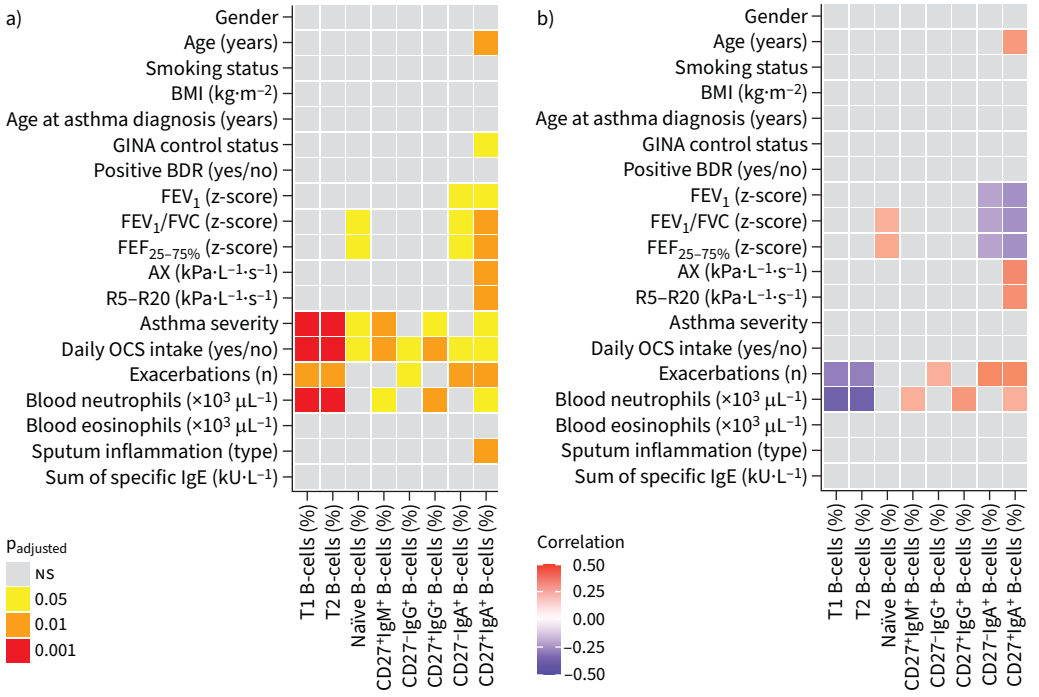

FIGURE 1 Pairwise a) comparisons and b) correlations between B-cell populations and clinical parameters of asthma patients and healthy controls. Colour code shows a) significant p-values and b) estimate for significant correlations analysed by Kruskal–Wallis or Spearman's correlation, respectively, with adjustment for multiple testing ($p_{adjusted}$). B-cell subsets are presented as percentage of total CD19$^+$ B-cells. BMI: body mass index; GINA: Global Initiative for Asthma; BDR: bronchodilator response; FEV$_1$: forced expiratory volume in 1 s; FVC: forced vital capacity; FEF$_{25-75\%}$: forced expiratory flow at 25–75% of FVC; AX: reactance area; R5–R20: resistance at 5 Hz−resistance at 20 Hz; OCS: oral corticosteroids; T1: transitional 1; T2: transitional 2. NS: nonsignificant.

associated with sputum eosinophilia, but not with blood eosinophilia or atopy (figure 2c and supplementary table S3).

Impaired lung function is associated with increased CD27$^+$IgA$^+$ memory B-cell frequency

Several lung function parameters indicative for airway obstruction were moderately associated with distinct B-cell patterns. Increased frequencies of IgA$^+$ memory B-cells were associated with central airway obstruction measured by forced expiratory volume in 1 s (FEV$_1$) and FEV$_1$/forced vital capacity (FVC) and small airway obstruction measured by forced expiratory flow at 25–75% of FVC (FEF$_{25-75\%}$) as well as IOS parameters reactance area (AX) and resistance at 5 Hz−resistance at 20 Hz (R5−R20) (figure 2d, supplementary figure S2c and supplementary table S3).

Association of IgA$^+$ memory B-cells and airway obstruction is independent from OCS treatment

As already presented, regular treatment with OCS showed a significant association with all investigated B-cell populations (figure 1 and supplementary table S3). We chose a linear model to investigate if any of the associations seen in figure 1 remained significant independently of OCS intake and age (table 3).

The linear model confirmed the association of increased percentages of CD27$^+$IgA$^+$ memory B-cells with SAD which was independent from OCS intake and age. The OCS-independent association was strongest between CD27$^+$IgA$^+$ B-cells and the IOS parameters AX ($p=3.3\times10^{-7}$) and R5−R20 ($p=7.2\times10^{-5}$), both indicating small airway obstruction (table 3). Comparing the association between R5−R20 and CD27$^+$IgA$^+$ B-cells in the linear model between patients with and without regular OCS intake showed no significant difference in the slope describing the association ($p=0.148$); however, percentages of IgA$^+$ memory B-cells were overall higher in patients with OCS (supplementary figure S3).

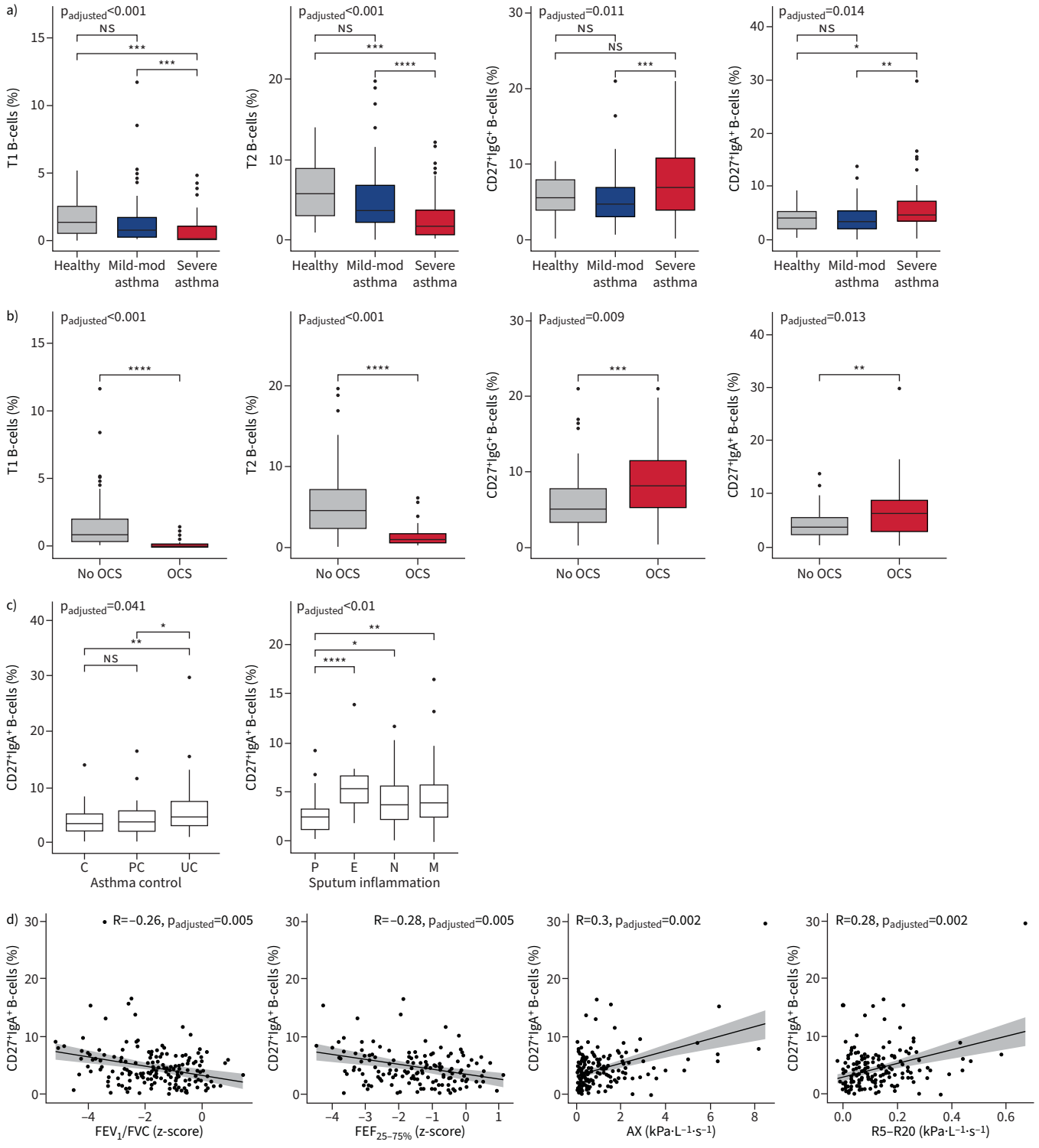

FIGURE 2 Associations between B-cell subsets and clinical parameters of asthma patients and healthy controls. Associations with a) asthma severity, b) oral corticosteroid (OCS) intake, c) asthma control and sputum inflammation, and d) forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC), forced expiratory flow at 25–75% of FVC ($FEF_{25–75\%}$), reactance area (AX) and resistance at 5 Hz–resistance at 20 Hz (R5–R20) are shown for asthma patients and healthy controls. Overall adjusted p-values after multiple test corrections and p-values from categorical group comparisons are shown as well as R and adjusted p-values from Spearman correlations. Further significant associations are shown in supplementary figure S2. T1: transitional 1; mod: moderate; T2: transitional 2; OCS: oral corticosteroids; C: controlled; PC: partly controlled; UC: uncontrolled; P: paucigranulocytic; E: eosinophilic; N: neutrophilic; M: mixed granulocytic. NS: nonsignificant; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

TABLE 3 Linear model

B-cell variable	Clinical variable	Independent variables per model				q-value of clinical variable
		Term	Estimate	SE	p-value	
$CD27^+IgA^+$ memory B-cells	AX	AX	0.886	0.167	<0.001	<0.001
		Age	0.034	0.016	0.039	
		Regular OCS	2.001	0.624	0.002	
		(Intercept)	1.358	0.869		
$CD27^+IgA^+$ memory B-cells	R5–R20	R5–R20	9.117	2.240	<0.001	0.002
		Age	0.031	0.017	0.066	
		Regular OCS	2.156	0.627	0.001	
		(Intercept)	1.307	0.896		
$CD27^+IgA^+$ memory B-cells	$FEF_{25–75\%}$ (z-score)	$FEF_{25–75\%}$ (z-score)	−0.683	0.225	0.003	0.026
		Age	0.046	0.019	0.020	
		Regular OCS	2.154	0.713	0.003	
		(Intercept)	0.764	1.097		
$CD27^+IgA^+$ memory B-cells	FEV_1 (z-score)	FEV_1 (z-score)	−0.541	0.177	0.003	0.026
		Age	0.049	0.017	0.005	
		Regular OCS	2.137	0.663	0.002	
		(Intercept)	0.902	0.940		
$CD27^+IgA^+$ memory B-cells	FEV_1/FVC (z-score)	FEV_1/FVC (z-score)	−0.597	0.192	0.002	0.026
		Age	0.044	0.017	0.010	
		Regular OCS	2.279	0.649	0.001	
		(Intercept)	0.815	0.944		
$CD27^-IgA^+$ memory B-cells	Number of severe exacerbations	Number of severe exacerbations	0.223	0.068	0.001	0.026
		Age	0.0003	0.011	0.975	
		Regular OCS	0.613	0.475	0.199	
		(Intercept)	2.097	0.591		
$CD27^-IgA^+$ memory B-cells	AX	AX	0.339	0.111	0.003	0.026
		Age	−0.003	0.011	0.803	
		Regular OCS	0.997	0.415	0.017	
		(Intercept)	2.173	0.578		
$CD27^-IgA^+$ memory B-cells	R5–R20	R5–R20	4.224	1.448	0.004	0.032
		Age	−0.004	0.011	0.718	
		Regular OCS	1.152	0.406	0.005	
		(Intercept)	2.084	0.579		

Linear model describing B-cell subpopulations as a function of clinical characteristics with oral corticosteroids (OCS) and age as confounders. Coefficient estimate, standard error and p-value are given for each term in the model. p-values for the clinical variables were corrected for multiple tests (q-value) and all significant results are shown (q<0.05). AX: reactance area; R5–R20: resistance at 5 Hz−resistance at 20 Hz; $FEF_{25–75\%}$: forced expiratory flow at 25–75% of FVC; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity.

Additional associations were found for $FEF_{25–75\%}$, FEV_1 and FEV_1/FVC (table 3). Furthermore, percentages of $CD27^-IgA^+$ B-cell frequencies were also associated with AX and R5–R20, and additionally with frequency of severe exacerbations. All other associations seen between B-cell populations were not independent from effects of OCS intake (supplementary table S4).

IgA^+ memory B-cells are increased in asthma patients with SAD

As shown by the linear model, lung function parameters indicative of peripheral airway obstruction showed a significant association with $CD27^+$ and $CD27^-IgA^+$ memory B-cells. The strongest association was seen for both IOS parameters AX and R5–R20, which measure airway distensibility and small airway obstruction, respectively. We were therefore interested to further investigate if these cells were also increased in patients with SAD. The IOS parameter R5–R20 has been shown to appropriately reflect resistance of the small airways [18], detect SAD in asthma patients [21] and corresponds well to important clinical outcomes of SAD in asthma patients [18, 21]. We consecutively used R5–R20 values above the upper limit of normal (95th centile) according to published reference equations [26] to define SAD in our cohort and to analyse its association with proportions of IgA^+ B-cells in more detail.

SAD was present in 42% (63 out of 152) of all asthma patients in our cohort. Of these, 43% (27 out of 63) had mild–moderate asthma and 57% (36 out of 63) had severe asthma. Further clinical characteristics of all asthma patients with and without SAD are specified in supplementary table S5.

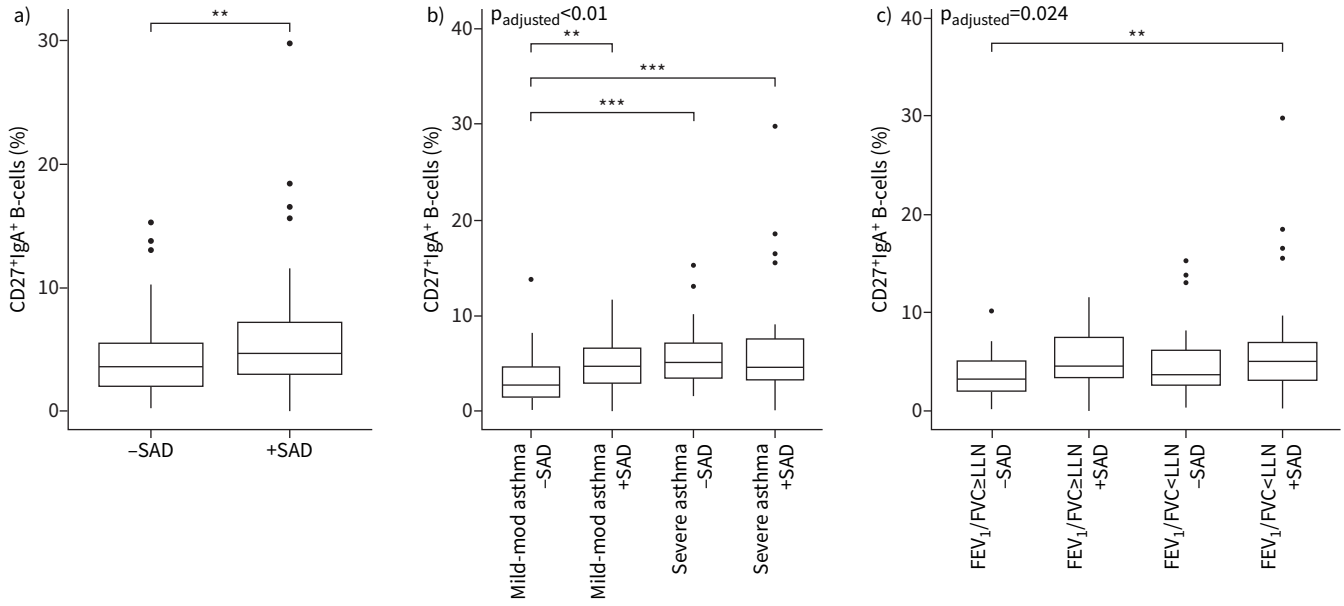

FIGURE 3 IgA$^+$ memory B-cells and small airway dysfunction (SAD). a) CD27$^+$IgA$^+$ memory B-cells in patients with or without SAD. b) CD27$^+$IgA$^+$ B-cells in patients with or without SAD in mild–moderate (mild-mod) asthma and severe asthma. c) CD27$^+$IgA$^+$ B-cells in patients with or without central airway obstruction (forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC)<lower limit of normal (LLN; z-score −1.64)) and with or without SAD. **: p<0.01; ***: p<0.001.

Percentages of CD27$^+$IgA$^+$ memory B-cells were increased in patients with SAD (figure 3a), while CD27$^-$IgA$^+$ memory B-cells did not show any differences (supplementary figure S4a). Furthermore, we observed differences in CD27$^+$IgA$^+$ memory B-cells depending on disease severity (figure 3b). IgA$^+$ memory B-cells were lowest in patients with mild–moderate asthma who did not have SAD and were significantly higher in mild–moderate asthma patients with SAD. Patients with severe asthma had overall increased percentages of IgA$^+$ B-cells without a difference between patients with and without SAD.

SAD and peripheral airway obstruction can occur independently from central airway obstruction. IgA$^+$ memory B-cells were only increased in patients with both central airway obstruction measured by FEV_1/FVC and small airway obstruction measured by IOS (figure 3c). In patients with normal FEV_1/FVC and SAD, the increase in the percentage of IgA$^+$ memory B-cells did not reach statistical significance compared with patients without central or peripheral airway obstruction (p=0.0662).

To investigate if IgA$^+$ memory B-cells were associated with SAD in the context of other known risk factors, including age, gender, BMI, smoking, markers of type 2 inflammation such as blood and sputum

TABLE 4 Regression model for small airway dysfunction (SAD) defined by resistance at 5 Hz–resistance at 20 Hz (R5–R20) in mild–moderate asthma patients

	Estimate	SE	p-value	95% CI lower bound	95% CI upper bound
CD27$^+$IgA$^+$ memory B-cell	0.29	0.109	0.008	1.087	1.683
Sputum eosinophils	0.095	0.04	0.017	1.022	1.197
Blood eosinophils	−4.805	2.015	0.017	0.0001	0.294
Gender (female)	−1.392	0.599	0.02	0.071	0.769
Sum of sIgE	0.004	0.002	0.13	0.998	1.008

Result of stepwise multivariate regression model (n=80). The dependent variable is SAD defined by the 95th centile of R5–R20. A stepwise-forward regression was used to find the best model using Akaike Information Criteria. The table shows the variables with best model fit (CD27$^+$IgA$^+$ memory B-cells (%), sputum eosinophils (%), blood eosinophils (×10^3 μL^{-1}), gender and sum of 36 allergen-specific IgE (sIgE; kU·L^{-1})). Variables not selected by best model fit are not shown (regular OCS intake (yes/no), exhaled nitric oxide fraction (ppb), body mass index (kg·m^{-2}), age (years) and smoking (pack-years)).

eosinophils, specific IgE, and F_{ENO} [20], we used a multivariate regression model. In patients with mild–moderate asthma, IgA^+ memory B-cells were associated with SAD in addition to other known risk factors such as sputum eosinophils and gender (table 4). Adding severe asthma patients to the model did not show an association between SAD and IgA^+ memory B-cells (supplementary table S6).

IgA^+ memory B-cells and clinical features of SAD

Patients with SAD present more often with uncontrolled asthma [28], frequent exacerbations [21] and impaired quality of life [18]. Percentages of $CD27^+IgA^+$ and $CD27^-IgA^+$ B-cells correlated with the number of exacerbations similarly as sputum eosinophils (figure 4 and supplementary table S7). Equally, $CD27^+IgA^+$ B-cells were correlated with impaired asthma control and reduced quality of life as assessed by the Asthma Control Questionnaire (ACQ-7) and Asthma Quality of Life Questionnaire, respectively (supplementary figure S5, and supplementary tables S8 and S9).

Since IgA^+ B-cells play an important role in mucosal immune defence, we analysed IgA^+ memory B-cells in SAD patients with frequent (two or more per year) respiratory tract infections (RTIs). In the ALLIANCE cohort, patients with and without SAD did not differ regarding the occurrence of frequent RTIs (17 out of 63 patients with SAD reported frequent RTIs compared with 17 out of 89 without SAD and with frequent RTIs; p=0.341). Equally, in patients with SAD, frequencies of IgA^+ memory B-cells did not differ depending on the presence or absence of frequent RTIs (supplementary figure S6).

Discussion

In this study we demonstrated significant alterations of immature and mature B-cell populations in asthma. Importantly, we described for the first time an unappreciated connection of IgA^+ memory B-cells with SAD. IgA^+ memory B-cells were associated with peripheral airway obstruction measured by R5–R20 irrespective of disease severity, and correlated with an increased number of severe exacerbations and worse asthma control.

OCS intake is known to be a major confounder of systemic immune responses, an effect that was evident for most B-cell populations examined in our study. Importantly, the link between increased systemic IgA^+

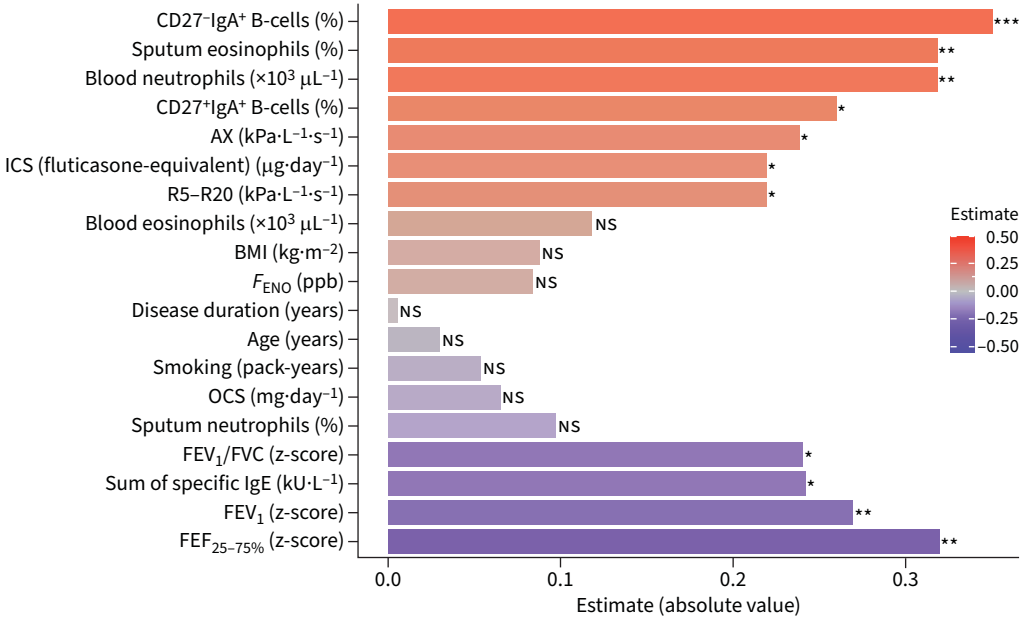

FIGURE 4 Correlations of number of exacerbations with clinical and B-cell parameters. Dark red defines the highest positive correlation between the parameters and dark blue shows the lowest negative correlation between the variables. Adjusted p-values after multiple test corrections are shown next to the bars. AX: reactance area; ICS: inhaled corticosteroids; R5–R20: resistance at 5 Hz–resistance at 20 Hz; BMI: body mass index; F_{ENO}: exhaled nitric oxide fraction; OCS: oral corticosteroids; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; $FEF_{25-75\%}$: forced expiratory flow at 25–75% of FVC. NS: nonsignificant; *: p<0.05; **: p<0.01; ***: p<0.001.

memory B-cell frequencies and lung function parameters indicative for peripheral airway obstruction such as R5−R20, AX and $FEF_{25–75\%}$ [18, 21] was independent of the influence of OCS intake and age. R5−R20 has been shown to reflect increased narrowing of the small airways [18, 21] and important clinical outcomes [18, 29]. We therefore used published reference equations for R5−R20 to define SAD and demonstrated an increase of IgA^+ B-cells in patients with SAD. This link was particularly evident in patients with mild–moderate asthma, indicating a role for IgA^+ B-cells in SAD irrespective of disease severity. This is important as SAD is not only found in severe asthma patients but also in mild–moderate disease [20, 21].

Little is known so far about inflammation or remodelling processes in the peripheral airways in asthma mostly due to their difficult accessibility. Histology data originate mostly from patients with fatal asthma attacks [30], limiting translation to asthma patients in general. There is some evidence for a role of type 2 inflammation in SAD from *in vitro* experiments [31] and histology of transbronchial biopsies revealed eosinophilic inflammation, particularly in the parenchyma of patients with nocturnal asthma symptoms [32], which are symptoms that are connected to SAD [28]. Clinical markers of type 2 inflammation, *e.g.* blood and sputum eosinophils, have also been linked to the presence of SAD [20] and type 2-targeting biologicals have been shown to ameliorate peripheral airway resistance measured by R5−R20 [33]. However, overall knowledge about inflammation connected to SAD is still very limited, particularly also regarding the impact of B-cells on SAD in asthma patients.

While our study is the first to link IgA^+ memory B-cells with SAD in asthma, our finding is supported by several studies linking IgA^+ B -cells to small airway inflammation in COPD. Histology studies of lungs from patients with COPD show increased IgA^+ B-cell frequencies in lymphoid follicles, particularly in the distal lung parenchyma and close to small airways, which correlate with disease severity [16]. Furthermore, in COPD there is a strong link between localised mucosal deficiency of secretory IgA (sIgA) and increased inflammation and airway remodelling most likely driven by impaired local pathogen defence [17, 34]. Additionally, reduced capacity for transcytosis of IgA over the epithelial barrier has been shown in both small airways of COPD patients [34] and airway epithelial cells in asthma [35], and sIgA in bronchoalveolar lavage fluid inversely correlates with asthma symptoms [36].

However, it remains unsolved if the increased presence of IgA^+ B-cells in the lung periphery of COPD patients with small airway disease reflects an exacerbated response against pathogens, potentially due to intraluminal sIgA deficiency, or if they could drive inflammation and remodelling, *e.g.* by producing antibodies against self-antigens [16].

Here, we showed for the first time that SAD in asthma patients is associated with increased frequencies of circulatory IgA^+ memory B-cells. This is in concordance with previous observations showing that systemic and pulmonary memory B-cell pools are connected, as memory B-cells in the lung depend on both local induction [37] as well as replenishment from extrapulmonary organs [38, 39]. Furthermore, we carefully investigated and excluded other reasons for increased blood IgA^+ memory B-cells in the context of asthma, *e.g.* frequent RTIs, smoking [40] and atopy [41].

Based on our analysis and previously published histological evidence [30], IgA^+ memory B-cells could serve as a biomarker for inflammation of the small airways, a compartment of the lung that is difficult to reach for diagnostic evaluation especially in asthma patients in whom lung histology is usually not available. However, due to the observational character of the ALLIANCE cohort, we cannot answer the question whether increased IgA^+ memory B-cells are a cause or co-phenomenon of SAD. Future studies need to confirm this link and assess its use as a biomarker for SAD development and progression.

In addition to our results regarding IgA^+ memory B-cells, we demonstrate substantial changes of other B-cell populations in asthma. Naïve mature B-cells as well as T1 and T2 B-cells were reduced in patients with severe asthma compared with mild–moderate asthma patients, while IgG^+ and IgM^+ memory B-cells were increased in severe asthma. However, these findings did not remain significant after correction for regular OCS intake, a treatment which affected 58% of the severe asthma patients in our cohort demonstrating the importance of considering steroid effects in the analysis. Further differentiation between asthma-specific or steroid effects or a combination of both was therefore not possible for these B-cell populations, a problem that has been described by other authors as well, particularly with regard to early B-cell differentiation [42, 43]. Equally, the association seen between several B-cell populations and blood neutrophils in our dataset did not remain significant after correction for OCS, most likely also due to effects of OCS on neutrophil frequencies [44]. Noteworthy, we were able to uncover a significant and OCS-independent association between IgA^+ memory B-cells and SAD. Still, we cannot completely

exclude an additional effect of OCS on IgA^+ memory B-cell frequencies in patients with severe asthma. This could also explain why the multivariate model which compared IgA^+ memory B-cells to other known risk factors for SAD only revealed a significant association when focusing on patients with mild–moderate asthma who are not exposed to OCS or high inhaled doses of corticosteroids.

A particular strength of our study is the stringent statistical design. Treatment effects are an inevitable problem in asthma research since most patients are already under treatment at the time of inclusion into an observational study. Therefore, appropriate statistical measures need to be applied to control for OCS effects, which confirmed in our study a new and until now undescribed role of IgA^+ memory B-cells in asthma patients with SAD.

A weakness of our study is that we did not investigate B-cells in lung tissue or in the airways. Lung histology as used in COPD studies is rarely available for patients with asthma. Future studies should explore and correlate lung and blood IgA^+ memory B-cells using bronchoalveolar lavage fluid or sputum and ideally lung tissue in combination with additional support from experimental murine models [7]. Additionally, more data are needed regarding the predictive use of IgA^+ memory B-cells for SAD development.

In conclusion, we showed that B-cell populations are altered in asthma compared with controls, and differ between mild–moderate and severe asthma, and we described disease-specific changes in the B-cell repertoire that are independent from systemic corticosteroid effects.

Our results reveal a new and until now undescribed association of IgA^+ memory B-cells in asthma patients with SAD, an important clinical feature of asthma with significant impact on symptom burden and quality of life. Most importantly, our data highlight for the first time a role for IgA^+ B-cells in asthma and particularly in SAD even in milder disease stages. Future studies are needed to elucidate the specific effects of IgA^+ B-cells on the development of SAD, and to investigate the use of IgA^+ memory B-cells as a biomarker for early diagnosis of SAD in asthma and prevention of lung function decline.

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