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Letter to the Editor

Allergic asthmatic subjects have elevated levels of IgE+B-cells in the airways

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KEY WORDS

allergy, asthma, B cells, IgE, sputum

DISCLOSURE

This study did not receive funding. Authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

JPO, BMS, SGS, RS and GMG designed the experiments and provided technical input on the project.

JPO, CDO, CEM, TXS and RMW coordinated the clinical procedures. JPO, BMS, SP, CDO, CEM and

SGS handled the biological specimens. JPO analyzed the data and prepared figures and tables for the

manuscript. JPO, SP, RS and GMG completed the writing of the manuscript.

KEY MESSAGES

- The levels of B cells, IgE+ B cells, IgE+ memory B cells and IgE+ plasma cells are higher in the airways, but not in the blood of allergic asthmatic subjects compared to non-asthmatic controls.
- Airway IgE+ B cells are positively correlated with airway eosinophils and total IgE levels.

To The Editor:

Allergic asthma is an IgE-mediated disease whereby inhaled allergens cross-link membrane-bound IgE on the surfaces of mast cells and basophils, inducing the activation and release of pro-inflammatory mediators, and driving type-2 inflammation and the manifestation of clinical symptoms¹. Since B-cells solely produce IgE, it is evident that these cells play a crucial role in initiating allergen-induced inflammatory processes.

The level of IgE+B-cells in the airways of asthmatics has not been examined previously. There have been reports of increased allergen-specific IgE in the blood of allergic asthmatics approximately one month after whole-lung allergen challenge², and in bronchoalveolar lavage fluid 24-hours after segmental allergen challenge³, suggesting allergen exposure to the airways induces an acute localized accumulation of allergen-specific IgE, followed by a persistent systemic increase. We sought to quantify the level of IgE+B-cells in the airways and blood of subjects with allergic asthma to delineate their role in local inflammation in the airways.

The three groups of subjects recruited for the study were all 18 to 65 years of age and non-smokers with either allergic asthma (n=18), or allergies without asthma (n=19), or they were healthy controls without allergies or asthma (n=10) (Table E1). No subjects required medication for treatment of asthma or allergies and all subjects provided written informed consent. Each subject provided a sample of blood and induced sputum. Sputum cells were extracted from mucus plugs, and surface-stained with antibodies to CD45, CD19, CD27, CD138, or CD4, CD8; while peripheral blood mononuclear cells (PBMCs) were isolated from blood were surface-stained with antibodies to CD45, CD19, CD27, CD138, CD38. Sputum cells and PBMCs were stained intracellularly with antibodies to IgE and IgG. Cells were acquired with a Becton Dickinson LSR II flow cytometer, and cell analyses were completed using FlowJo software. The total IgE level in serum, and total IgE and B cell activating factor (BAFF) in sputum fluid-phase were measured using a commercially available ELISA kit. The

subject criteria, sample processing, cell staining procedures and gating strategies are detailed in the Supplementary Methods and Figure E1. All data are presented as mean $\pm$ SEM.

When expressed as a percentage of total lymphocytes, the frequency of B-cells in the sputum of asthmatic subjects was 11.8 $\pm$ 2.0%, which was over 2-fold higher than 5.4 $\pm$ 0.9% in allergic subjects and 4.9 $\pm$ 0.6% in controls (Figure 1A, $p<0.05$). The frequency of IgE+B-cells expressed as a percentage of total B-cells in sputum was 26.2 $\pm$ 3.2% in asthmatic subjects, which was over 3-fold higher than 7.3 $\pm$ 1.8% in allergic subjects and 4.5 $\pm$ 1.4% in controls (Figure 1B, $p<0.05$). Furthermore, the frequency of IgE+memory B-cells and IgE+plasma cells, expressed as a percentage of total B-cells in sputum was also higher in asthmatic subjects compared to the non-asthmatic subjects (Figure 1C-D, $p<0.05$). The level of sputum BAFF was elevated in asthmatic subjects compared to non-asthmatic subjects (Table E1),

In blood, there was no difference in the frequency of B-cells or IgE+B-cell subsets across groups (Figure 1E-H, $p>0.05$). When B-cells were expressed as absolute number of cells per-milliliter of blood, or per-gram of sputum, we found higher levels of IgE+B-cell subsets in the airways of asthmatic subjects compared to the other groups (Figure E2A-D, $p<0.05$), with no differences detected in blood (Figure E2E-H, $p>0.05$). In contrast, we did not observe any difference in the level of sputum memory B-cells, plasma cells, CD4+T-cells, CD8+T-cells or total lymphocytes between groups, nor was there a difference in blood between groups (Figure E3-E5, $p>0.05$). The frequency and absolute numbers of IgG+B-cells, evaluated as a control cell population, showed a higher tendency, but no statistical difference in asthmatics compared to the non-asthmatic subjects when measured in the sputum or blood (Figure E6, $p>0.05$). Furthermore, a positive correlation between IgE+ and IgG+B-cells were found in sputum (Figure E6C, $r=0.36$, $p=0.08$) and blood (Figure E6F, $r=0.39$, $p=0.03$).

In sputum, the frequency of IgE+B-cells positively correlated with eosinophils (Figure 2A, $r=0.65$, $p=0.0005$), sputum total IgE levels (Figure 2B, $r=0.68$, $p=0.0002$), and sputum BAFF levels (Figure 2C, $r=0.42$, $p=0.04$). The frequency of sputum B-cells also positively correlated with sputum

eosinophils (Figure 2D, $r=0.60$, $p=0.002$) and sputum BAFF levels (Figure 2F, $r=0.47$, $p=0.02$). There was no relationship between blood B-cells and IgE+B-cells, and plasma total IgE and blood eosinophils (Figure E7, $p>0.05$).

Kidney et al reported higher a sputum level of B-cells in asthmatics ($16.5\pm3.4\%$ of lymphocytes) compared to non-asthmatic smokers ($4.0\pm1.1\%$ of lymphocytes)⁴; however, no further reports on human B-cells have been published since. Using a more stringent panel of B-cell markers, the current study found similarly elevated levels of B-cells in asthmatic subjects ($11.8\pm2.0\%$ of lymphocytes), thereby corroborating previous findings.

Historically, IgE+B-cells have been difficult to detect in blood, however methodological advances including availability of fluorochrome-labeled antibodies and flow cytometers with more lasers has allowed for B-cell subsets to be measured. Recent papers have reported allergen-specific IgE+B-cells measured in the blood⁵, nasal biopsies and nasal lavage of patients with allergic rhinitis^{6,7}. With our methods we were able to consistently detect IgE+B-cells in the blood and sputum of our subjects, which allowed us to show, for the first time, significantly higher levels of IgE+B-cell subsets in the sputum of asthmatics compared to non-asthmatic controls. These findings, along with higher levels of sputum BAFF in our allergic asthmatic subjects and a positive correlation between sputum BAFF, and B-cells and IgE+B-cells, support a mechanism whereby local maturation and proliferation of B cells occur in the airways of allergic asthmatic subjects⁸, and together with IL-4 and IL-13⁹, drives local increases in IgE+B-cell subsets and IgE levels.

We also observed a positive correlation between sputum eosinophils, and B-cells and IgE+B cells, supporting the findings of Kidney et al that described a relationship between sputum eosinophils and B-cells⁴. We also found a positive correlation between sputum total IgE levels and IgE+B cells, indicating that IgE-secreting B-cells in the airways, but not in the blood, are directly related to IgE levels.

The results of this study highlight the close relationship between airway IgE+B-cells, local production of IgE, and the development of eosinophilic airway inflammation in allergic asthma. We propose that the findings of this study support the development of therapeutic strategies targeting IgE+B-cells for treatment of asthma.

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- 156
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158 LIST OF ABBREVIATIONS

159 AA - allergic asthmatic

160 ANA - allergic non-asthmatic

161 BAFF - B cell activating factor

162 FEV₁ - forced expiratory volume in 1 second

163 IgE - immunoglobulin E

164 IgG - immunoglobulin G

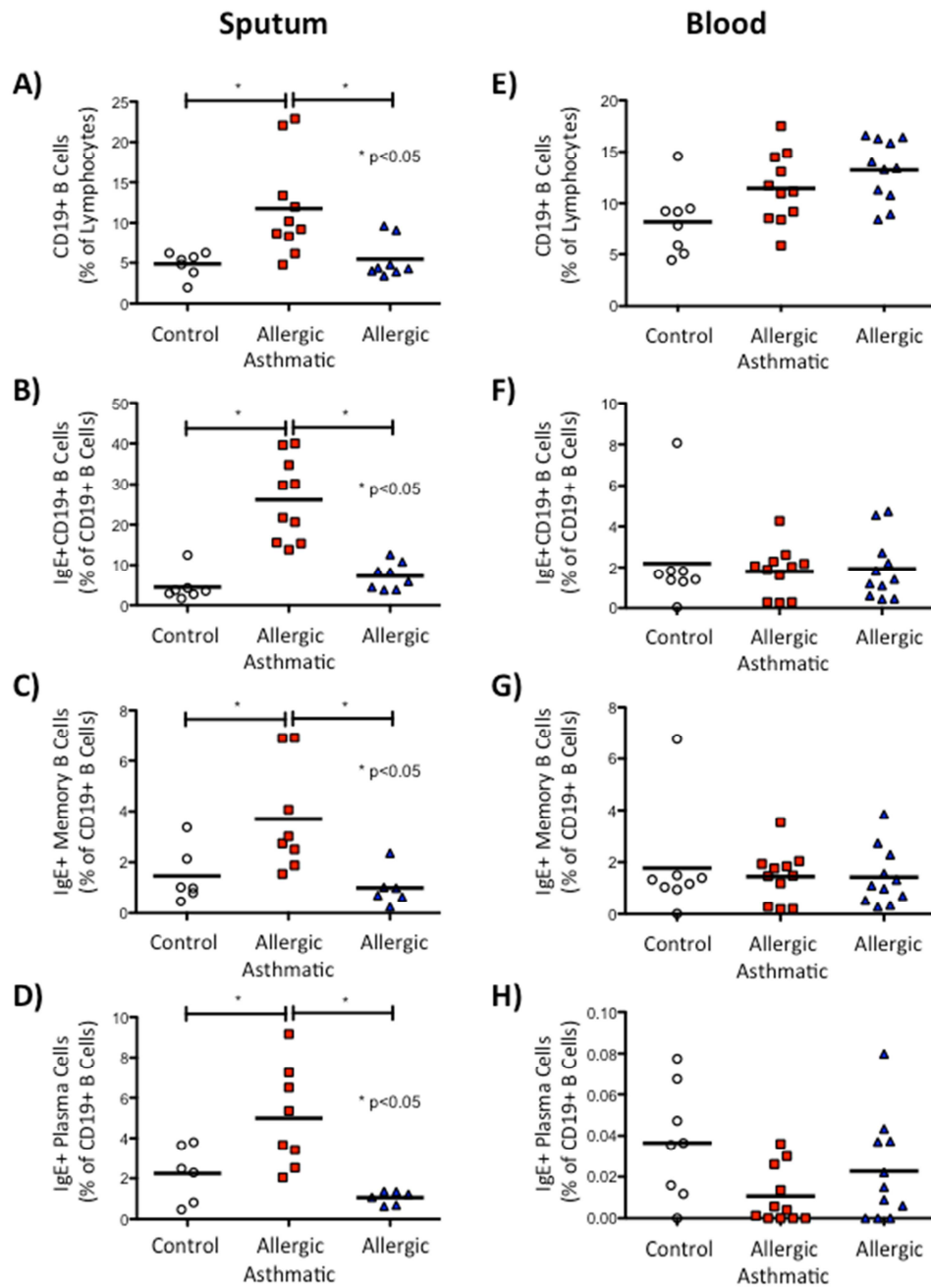
165 IL - interleukin

166 HC - healthy control

167 PC₂₀ - provocative concentration of methacholine resulting in a minimum of 20% fall in FEV₁

168

169 **FIGURES**



170

171 **Figure 1: The levels of B cells, IgE+ B cells, and IgE+ B cell subsets (memory B cells and plasma**

172 **cells) in the sputum and blood of subjects with allergic asthma and non-asthmatic controls. Data**

173 **were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean±SEM. In**

174 **sputum analysis of B cells and IgE+ B cells: control n=7, allergic asthmatic n=10, allergic n=8, while**

175 sputum analysis of IgE+ B cell subsets: control n=6, allergic asthmatic n=8, allergic=6. In blood
176 analysis of all cells: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are
177 denoted by *, where $p < 0.05$.

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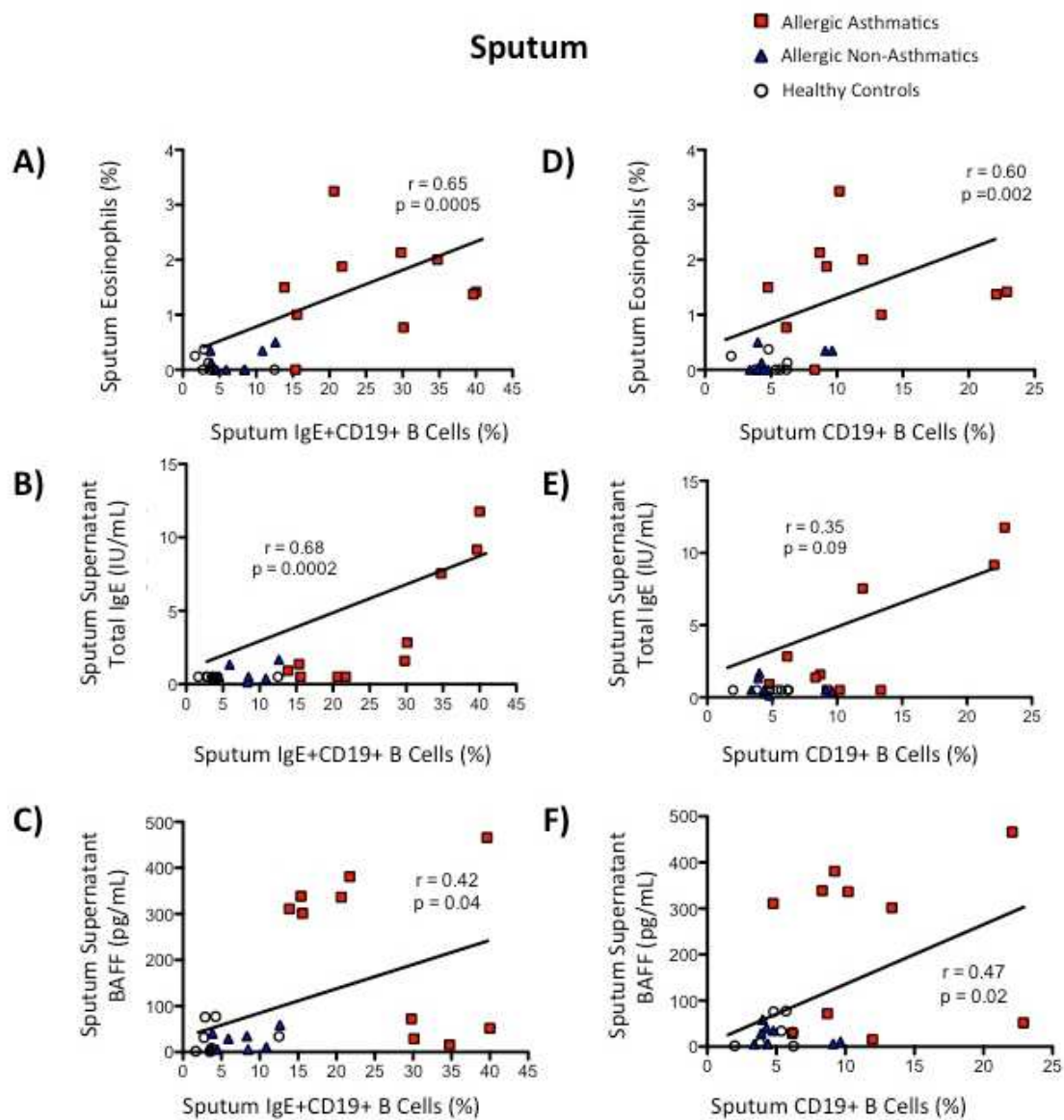
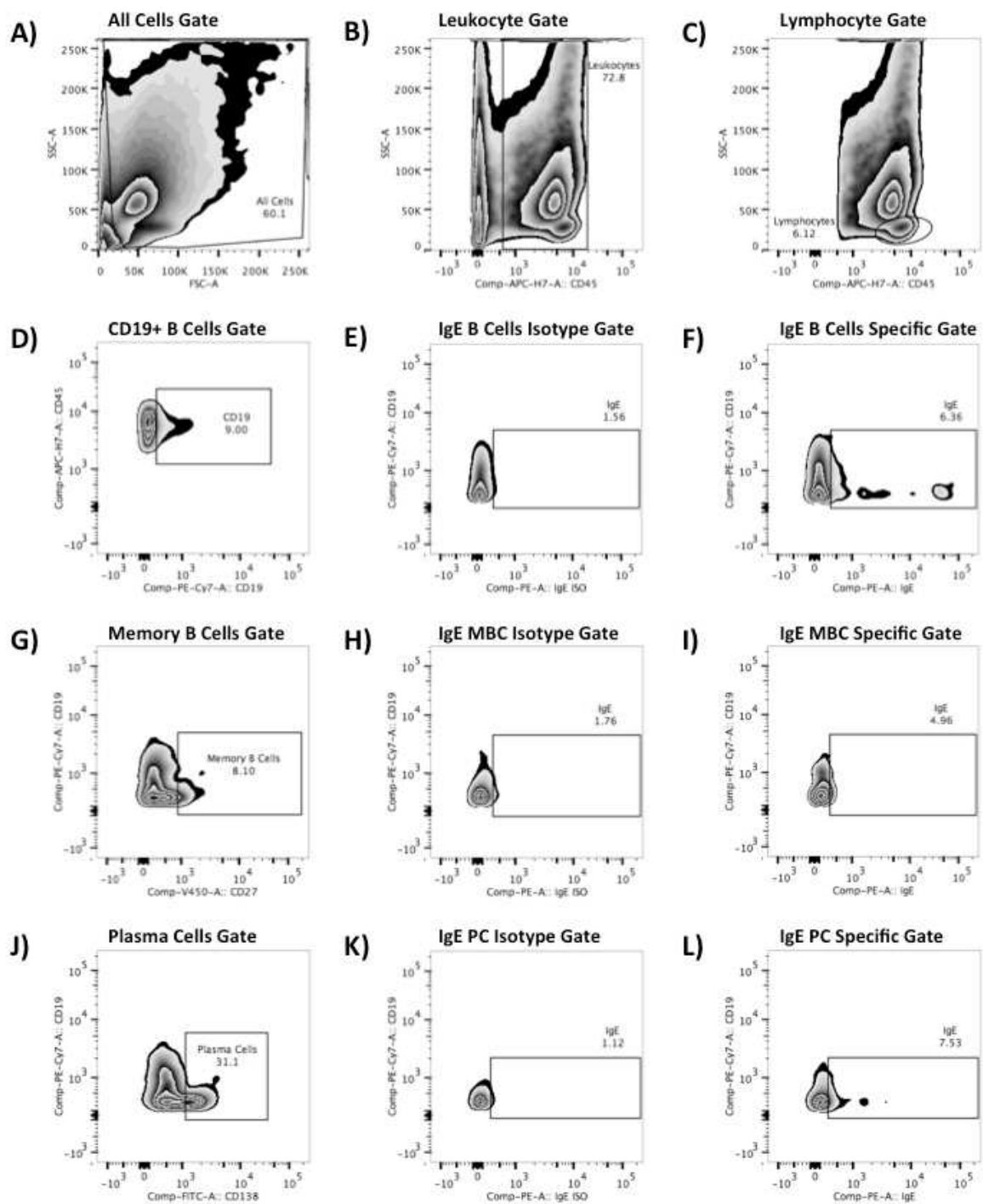
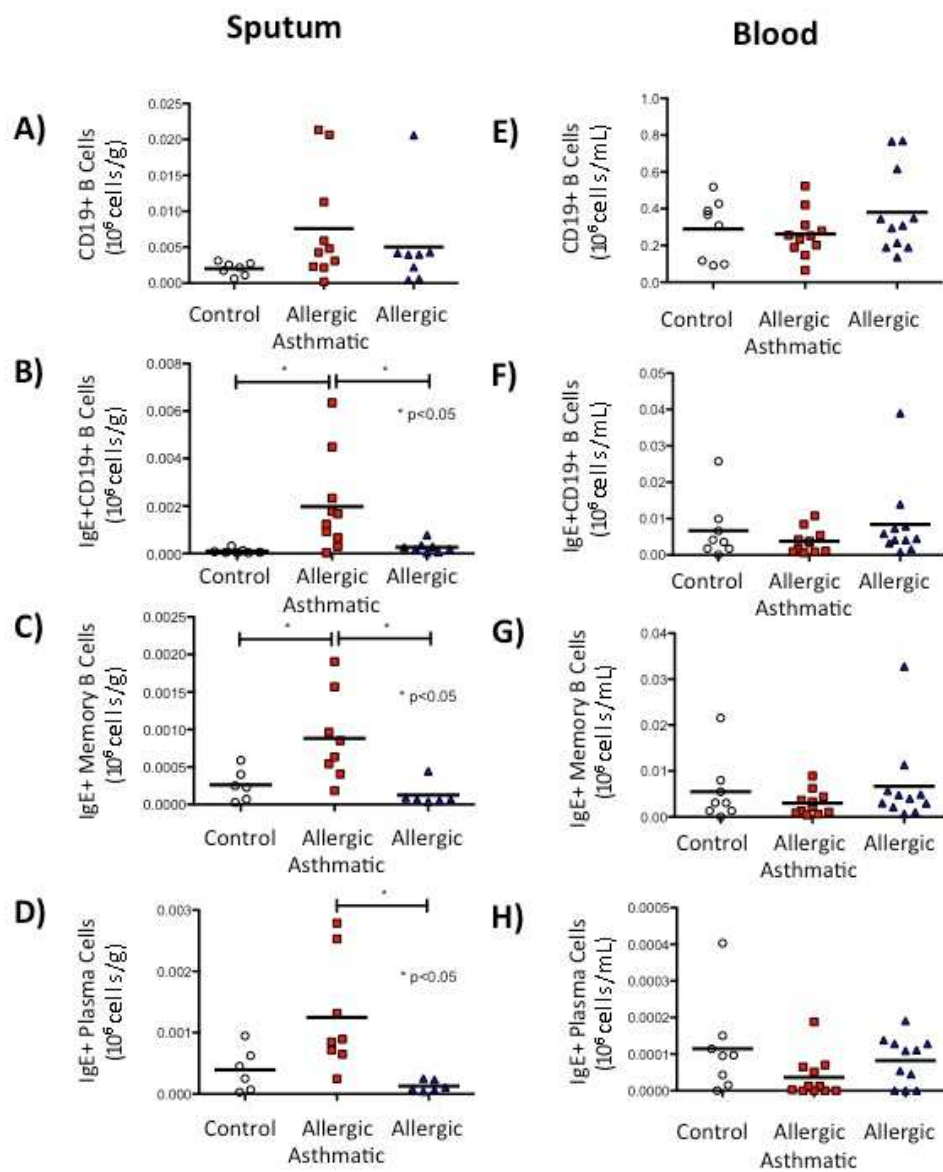
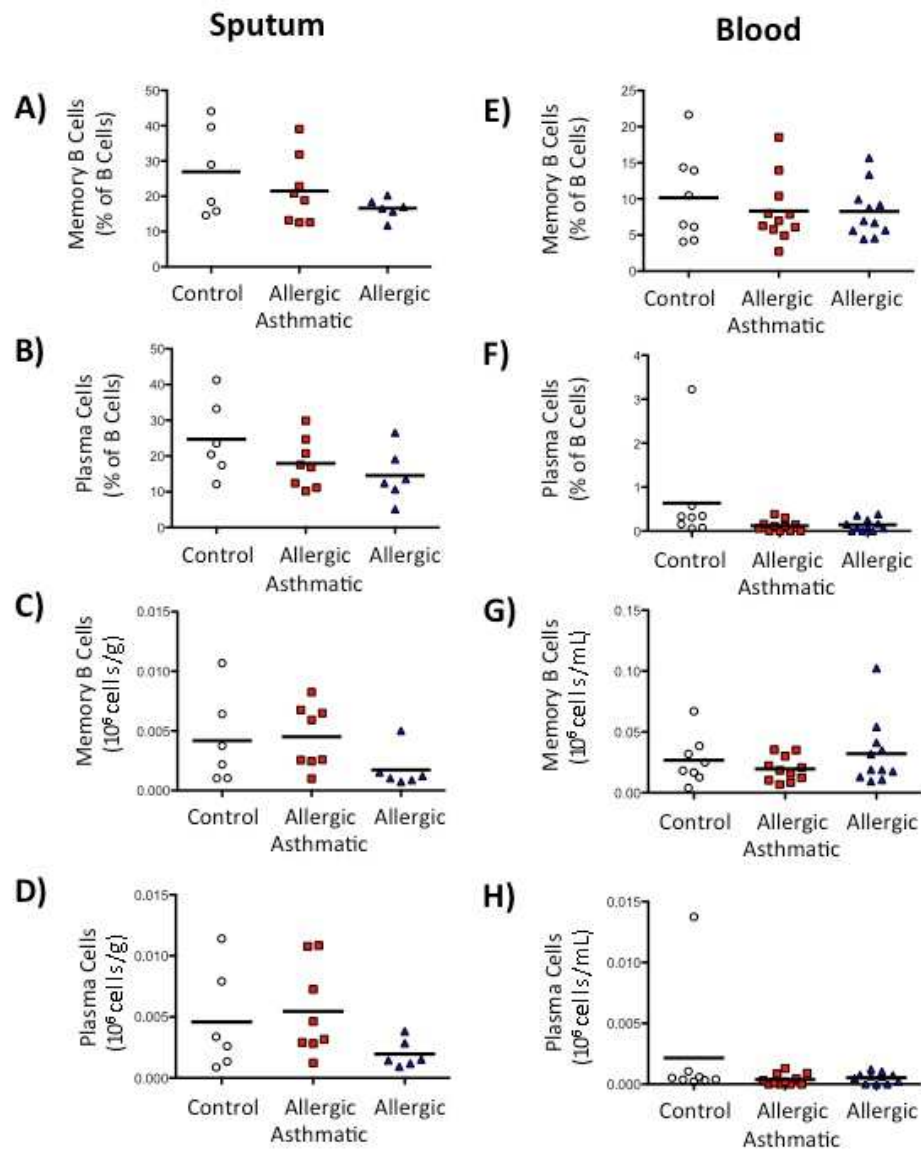
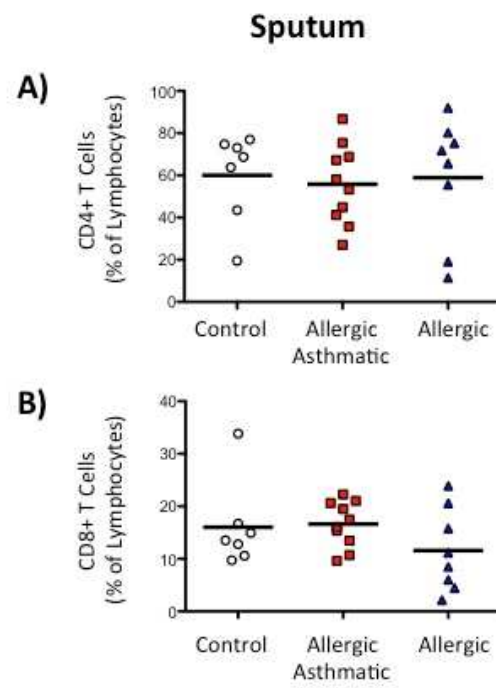


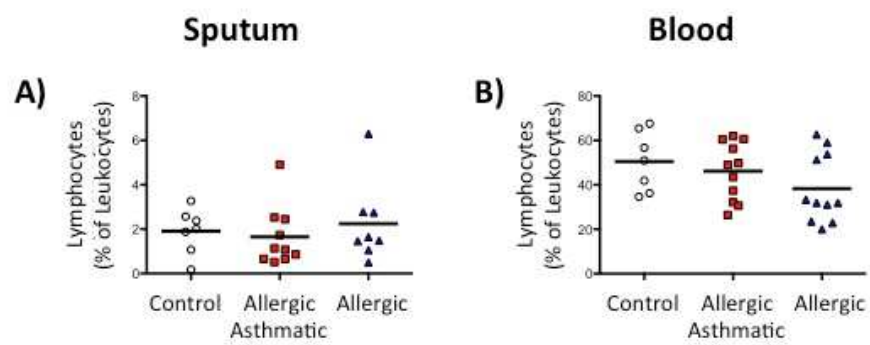
Figure 2: The relationship between B Cells and IgE+ B cells, and eosinophils and total IgE and B cell activating factor (BAFF) in sputum of subjects with allergic asthma and non-asthmatic controls. Data were correlated with Spearman rank r-values and linear regression lines of best fit. In sputum: control n=7, allergic asthmatic n=10, allergic n=8. Exact p-values were denoted, where $p < 0.05$ was considered a significant correlation.

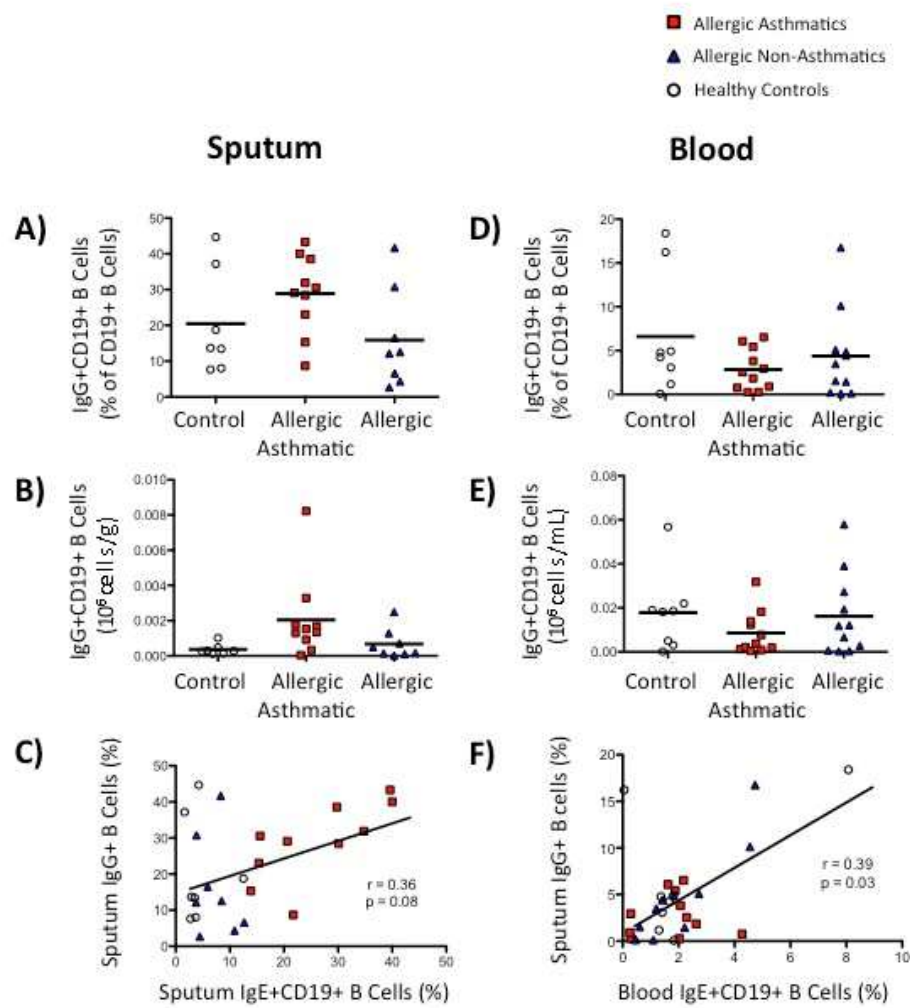


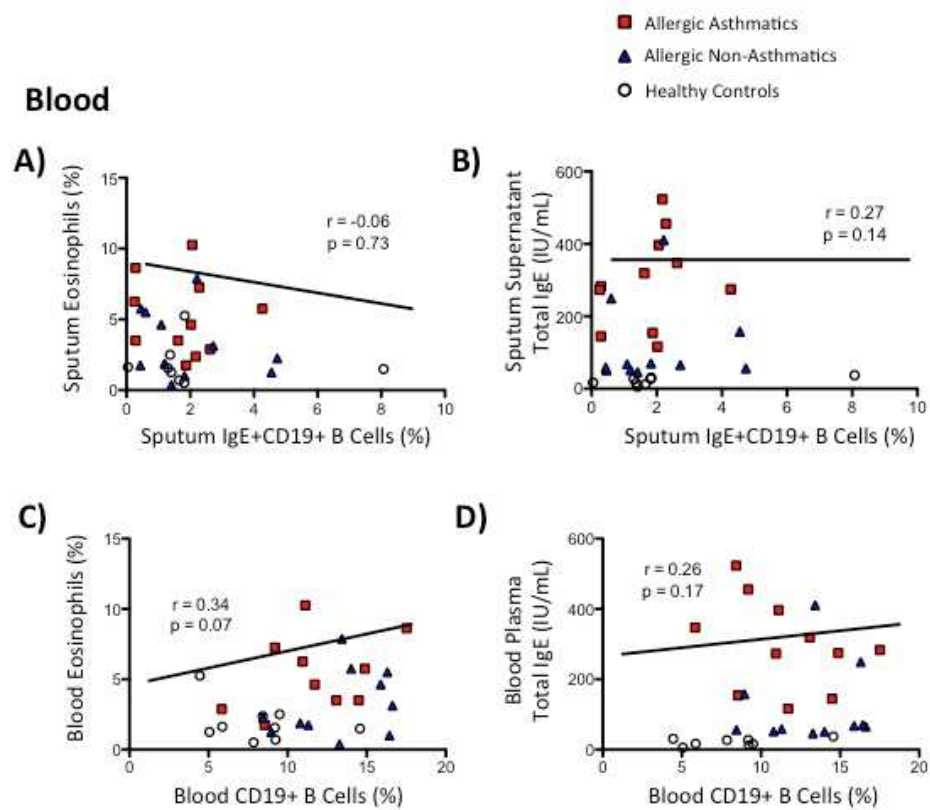












Supplementary Information**Allergic asthmatic subjects have elevated levels of IgE+B-cells in the airways**

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19 SUPPLEMENTARY METHODS

20 Subject Criteria and Study Design

21 Allergic asthmatics (n=18), allergic non-asthmatics (n=19) and healthy controls (n=10) all
22 between 18 and 65 years of age were recruited to participate in the study. No subjects required
23 medication for treatment of asthma or allergies. All allergic asthmatic subjects were atopic with mild
24 asthma; the percent predicted forced expiratory volume in one second (FEV₁) was $\geq 70\%$ of predicted,
25 and the methacholine provocative concentration causing at least a 20% fall in FEV₁ (PC₂₀) ≤ 16 mg/ml¹.
26 Subjects were excluded if they had asthmatic exacerbations or respiratory tract infections within four
27 weeks of the study start date, or underlying diseases other than asthma. Participating subjects
28 underwent a screening procedure to determine allergic status by skin prick test, and lung function by
29 spirometry. All subjects provided written informed consent. Subjects donated peripheral blood and
30 induced sputum.

31 Clinical Tests

32 Spirometry was performed following ATS guidelines². Skin prick test was performed using
33 common aeroallergens and histamine and saline as positive and negative controls, respectively. A
34 wheal diameter of >2 mm was considered to be positive³⁻⁵. A methacholine challenge test using tidal
35 breathing from a Wright Nebulizer was completed to determine the provocative concentration that
36 resulted in a 20% fall in FEV₁ (PC₂₀)⁶.

37 Sample Collection and Cell Processing

38 Sputum was induced by inhalation of 3, 4 and 5% saline and mucous plugs were selected and
39 processed as previously described^{7,8}. Briefly, sputum was treated with Dulbecco's phosphate buffered
40 saline (DPBS) and dithiothreitol (DTT) and filtered through a nylon mesh, the cells were pelleted by
41 centrifugation. Two cytopins were prepared and stained with Diff Quik for cell differentials and the
42 remaining cells were stored at 4°C flow cytometry staining. The fluid phase was frozen for
43 measurements of total IgE.

Peripheral blood was collected into vacutainers containing heparin. A total cell count was performed using a hemocytometer and smears were prepared and stained with Diff Quik for cell differentials. A portion of the blood was centrifuged at 1000 g for 10 minutes to collect plasma for measurement of total IgE levels. The remaining blood was diluted with 10% fetal bovine serum (FBS) Roswell Park Memorial Institute (RPMI) buffer, layered on AccuPrep density gradient, and then centrifuged at 800 g for 20 minutes without a break. The peripheral blood mononuclear cell (PBMC) layer was collected and stored at 4°C for flow cytometry staining.

Cell Staining and Gating Strategy for B Cells

Cells were stained with fluorochrome-conjugated antibodies for flow cytometry analysis of B cells and B cell subsets. Cells were initially washed twice with FACS buffer. Antibodies to surface markers were incubated with the cells for 30 minutes, and the cells were washed, fixed and permeabilized. Antibodies to intracellular markers were then incubated with the cells for 30 minutes. Appropriate isotypes were used as negative controls. The cells were acquired using a Beckton Dickinson (BD) LSRII flow cytometer (BD Bioscience, ON, CAN) and the files were analyzed with the FlowJo software (TreeStar, OR, USA).

Sputum cells were surface-stained with antibodies to CD45 (APC-H7), CD19 (PE-Cy7), CD27 (V450), CD138 (FITC), CD4 (APC-H7), CD8 (FITC) and intracellularly stained with IgE (PE) and IgG (APC). From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around all the cells, excluding debris. SSC and CD45 were then plotted and a gate was placed around the lymphocyte population (SSC^{low}CD45⁺). CD45 and CD19 were then plotted and a gate was placed around the CD19⁺ population. CD19⁺ B cells were used to plot CD19 with IgE and IgG to isolate IgE⁺ and IgG⁺ B cells in the airways. Furthermore, CD19⁺ B cells were used to plot CD19 with CD27 and CD138 to isolate memory B cells (MBCs) and plasma cells (PCs), respectively. IgE⁺ MBCs and PCs were also enumerated in the airways.

PBMCs were surface-stained with CD45 (APC-H7), CD19 (PE-Cy7), CD27 (V450), CD138 (FITC), CD38 (PE) and intracellularly with IgE (APC) and IgG (APC). The following was the gating strategy used to enumerate memory B cells (MBCs) (CD27+CD38-), plasmablasts (PCs) (CD27+CD38+) and plasma cells (PCs) (CD38+CD138+) from peripheral blood. From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around the lymphocyte population. SSC and CD19 were then plotted and a gate was placed around the CD19+ population. SSC and CD27 were plotted and a gate was placed around the CD27+ population. CD38 and CD27 were then plotted and this was used to obtain each of the required phenotypes, MBCs and PPCs. MBCs and PPCs were then used to plot CD19 with IgE and IgG isolate IgE+ and IgG+ MBCs and PPCs in circulation. Furthermore, CD19+ B cells were used to plot CD38 and CD138 and this was used to obtain PCs. PCs were then used to plot CD19 with IgE and IgG to isolate IgE+ and IgG+ PCs in blood.

For a representative gating strategy in sputum, refer to Figure E1.

Total IgE and BAFF Measurements

Total IgE (Abcam, ON, CAN) was measured in plasma and sputum fluid and BAFF (R&D Systems, MN, USA) was measured in sputum fluid phase using commercially available sandwich, enzyme linked immunosorbent assay (ELISA) kits..

Statistical Analyses

Data were presented as the mean±SEM with the exception of methacholine PC₂₀ presented as geometric mean (95% confidence interval). Airway physiology, sputum and blood cells (frequencies and magnitudes), and total IgE and BAFF levels were analyzed using 1-way ANOVA with Tukey post-hoc tests. In cases where total IgE in sputum fluid phase was not detected, we assigned the lower limit of detection (0.5 IU/mL). Spearman rank tests were used to evaluate the relationship between CD19+ B cells and IgE+ B cells, and eosinophils, total IgE, and BAFF in the airways. Significance was accepted at $p < 0.05$.

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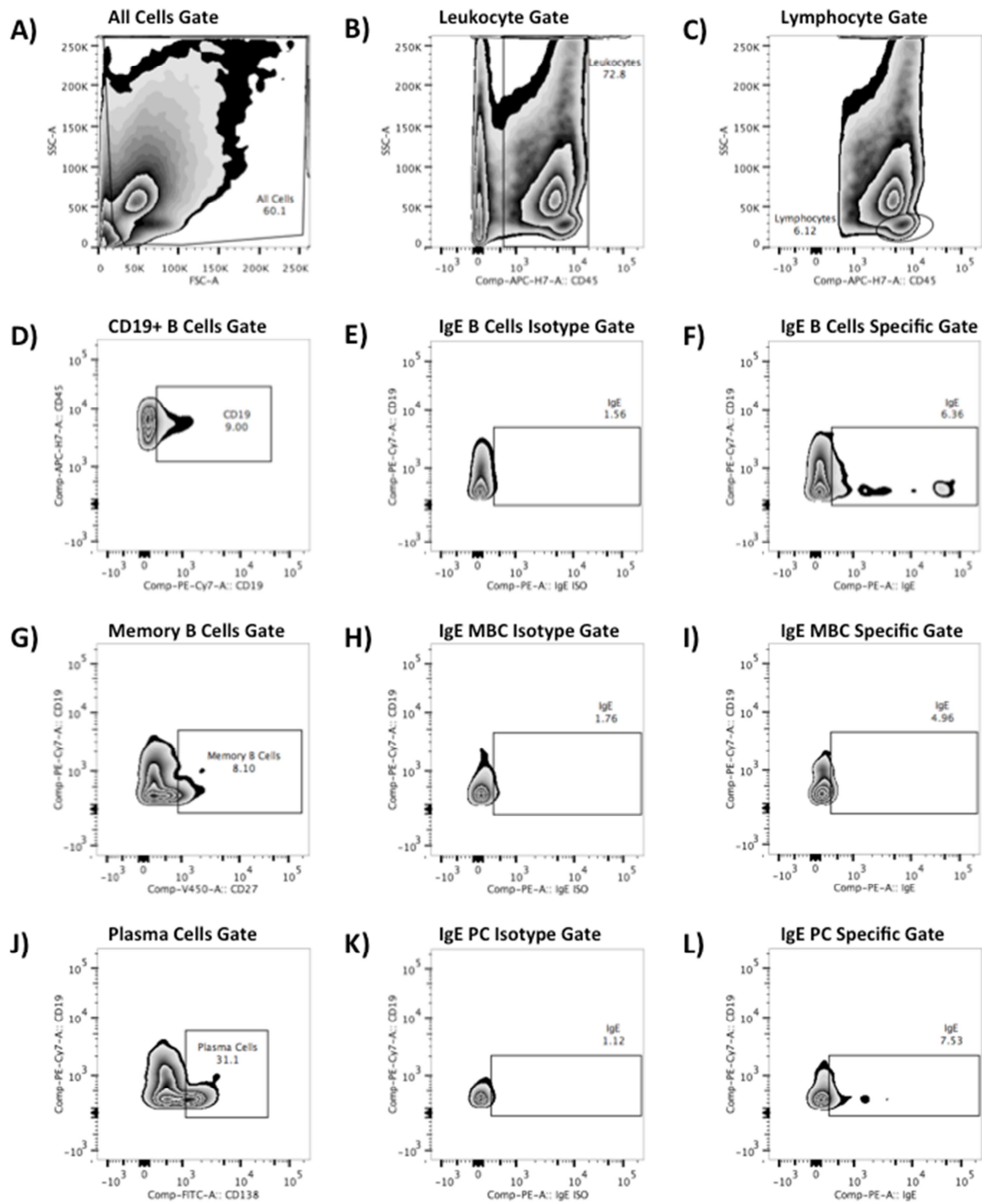
114 **SUPPLEMENTARY TABLES and FIGURES**

115 **Supplemental Table E1: Subject demographics.** Data are shown as mean±SEM, except
 116 methacholine PC₂₀, which is shown as geometric mean (95% CI). Significant findings are denoted by *
 117 where p<0.05 compared to healthy control subjects and † where p<0.05 compared to allergic non-
 118 asthmatic subjects.

119

	Controls (n=10)	Allergic Asthmatics (n=18)	Allergic (n=19)
Sex (M/F)	4/6	8/10	10/9
Age (years)	37.1±3.8	31.8±3.3	26.2±2.4
FEV₁ (% of predicted)	94.9±2.6	91.6±1.6	98.2±2.8
Methacholine PC₂₀ (mg/mL)	>16	3.7 (2.2, 6.4) *†	>16
Sputum Eosinophils (%)	0.08±0.04	1.8±0.3*†	0.6±0.1
Sputum Total IgE (IU/mL)	0.5±0.0	3.7±1.3*	0.8±0.2
Sputum BAFF (pg/mL)	32.8±12.3	230.3±53.4*†	24.0±7.0
Blood Eosinophils (%)	1.8±0.4	4.8±0.5*	3.2±0.4
Plasma Total IgE (IU/mL)	19.8±3.4	288.7±34.3*†	107.6±29.9
Skin test positive to:			
Cat Dander	0	15	12
Horse Dander	0	12	5
House Dust Mite	0	17	16
Trees	0	14	6
Grass	0	11	6
Ragweed	0	14	8

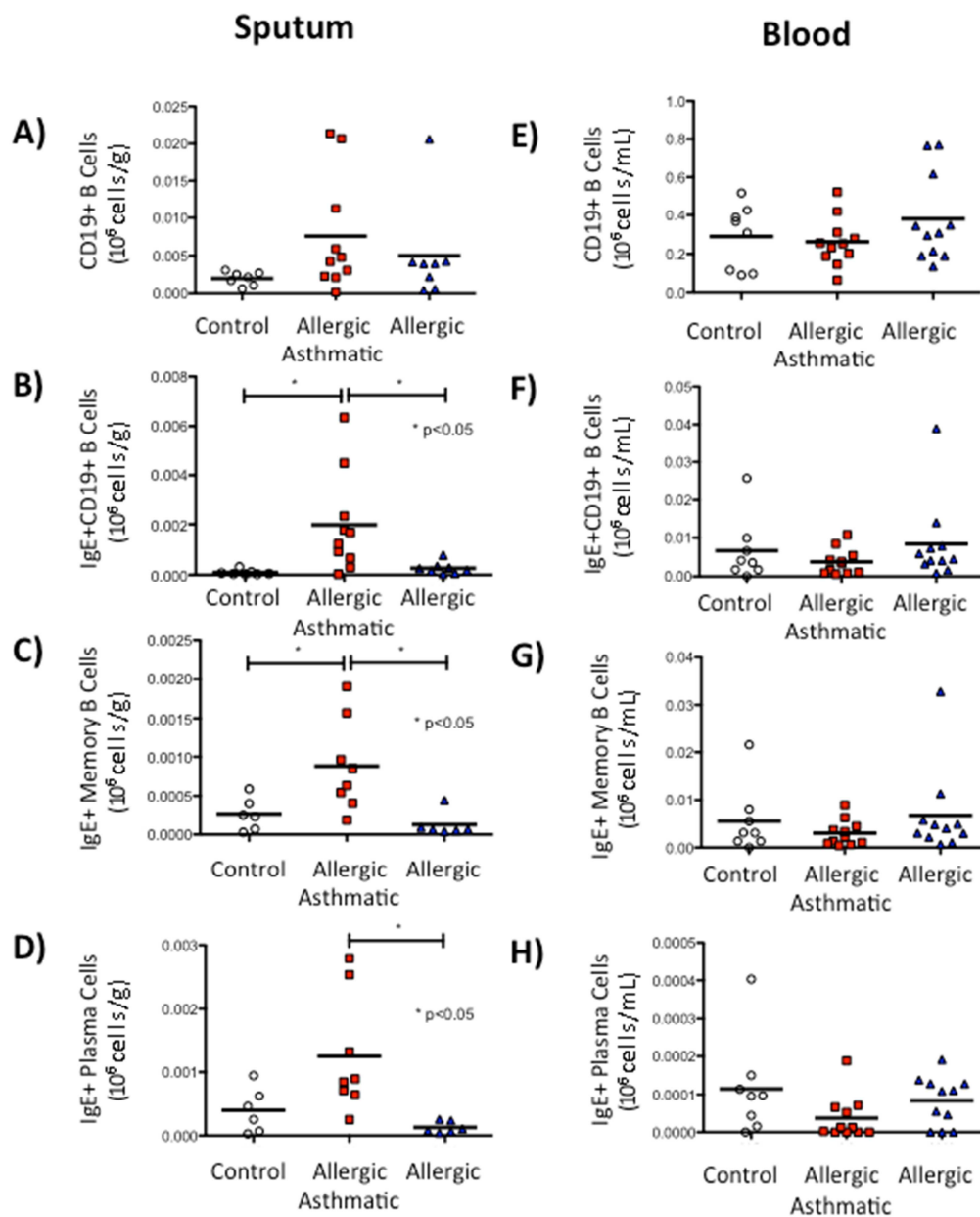
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122 Supplemental Figure E1: Representative gating strategy for IgE+ B cell subsets with isotype
123 controls in sputum.

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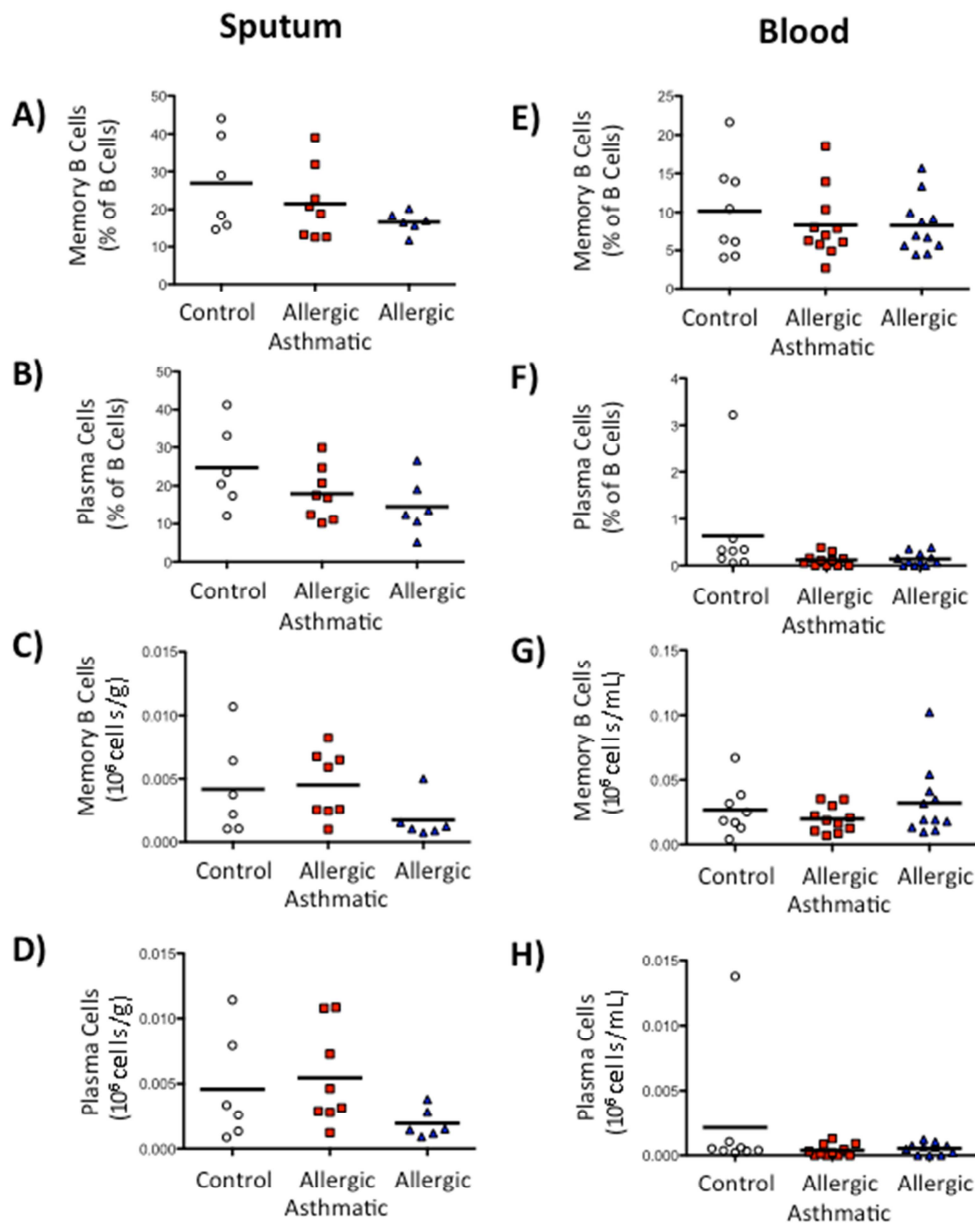
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126 **Supplemental Figure E2: The number of B cells, IgE+ B cells and IgE+ B cell subsets (memory B**
127 **cells and plasma cells) in the sputum and blood of subjects with allergic asthma and non-**
128 **asthmatic controls.** Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are
129 presented as mean $\pm$ SEM. In sputum analysis of B cells and IgE+ B cell numbers: control n=7, allergic
130 asthmatic n=10, allergic n=8, while sputum analysis of IgE+ B cell subset numbers: control n=6,

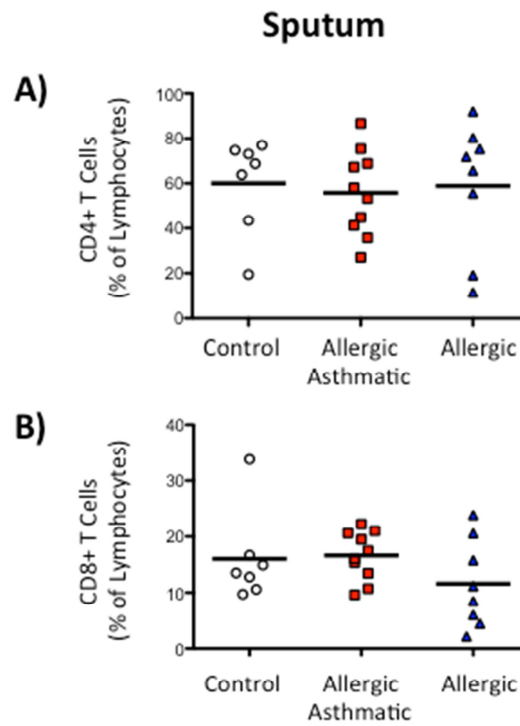
131 allergic asthmatic n=8, allergic=6. In blood analysis of all cell numbers: control n=11, allergic
132 asthmatic n=11, allergic n=8. Significant findings are denoted by *, where $p < 0.05$.

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ACCEPTED MANUSCRIPT



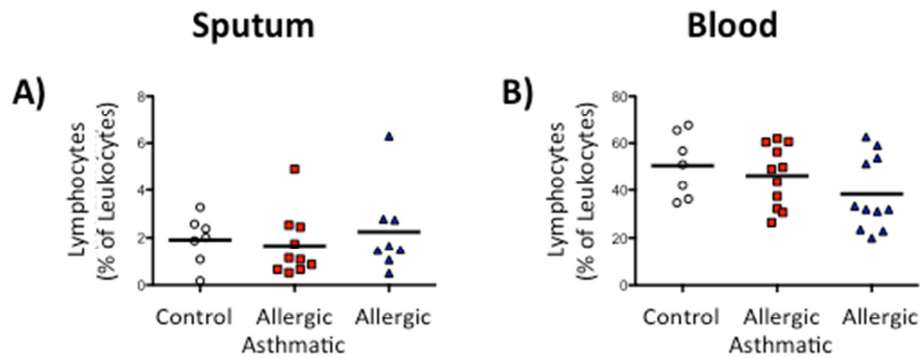
Supplemental Figure E3: The frequency and number of memory B cells and plasma cells in the sputum and blood of subjects with allergic asthma and non-asthmatic controls. Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean±SEM. In sputum: control n=6, allergic asthmatic n=8, allergic n=6. In blood: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are denoted by *, where $p < 0.05$.



141

142 **Supplemental Figure E4: The frequency of CD4+ T cells and CD8+ T cells in the sputum of**
 143 **subjects with allergic asthma and non-asthmatic controls.** Data were analyzed using a 1-way
 144 ANOVA with Tukey post-hoc tests and are presented as mean $\pm$ SEM. In sputum: control n=7, allergic
 145 asthmatic n=10, allergic n=8. Significant findings are denoted by *, where $p < 0.05$.

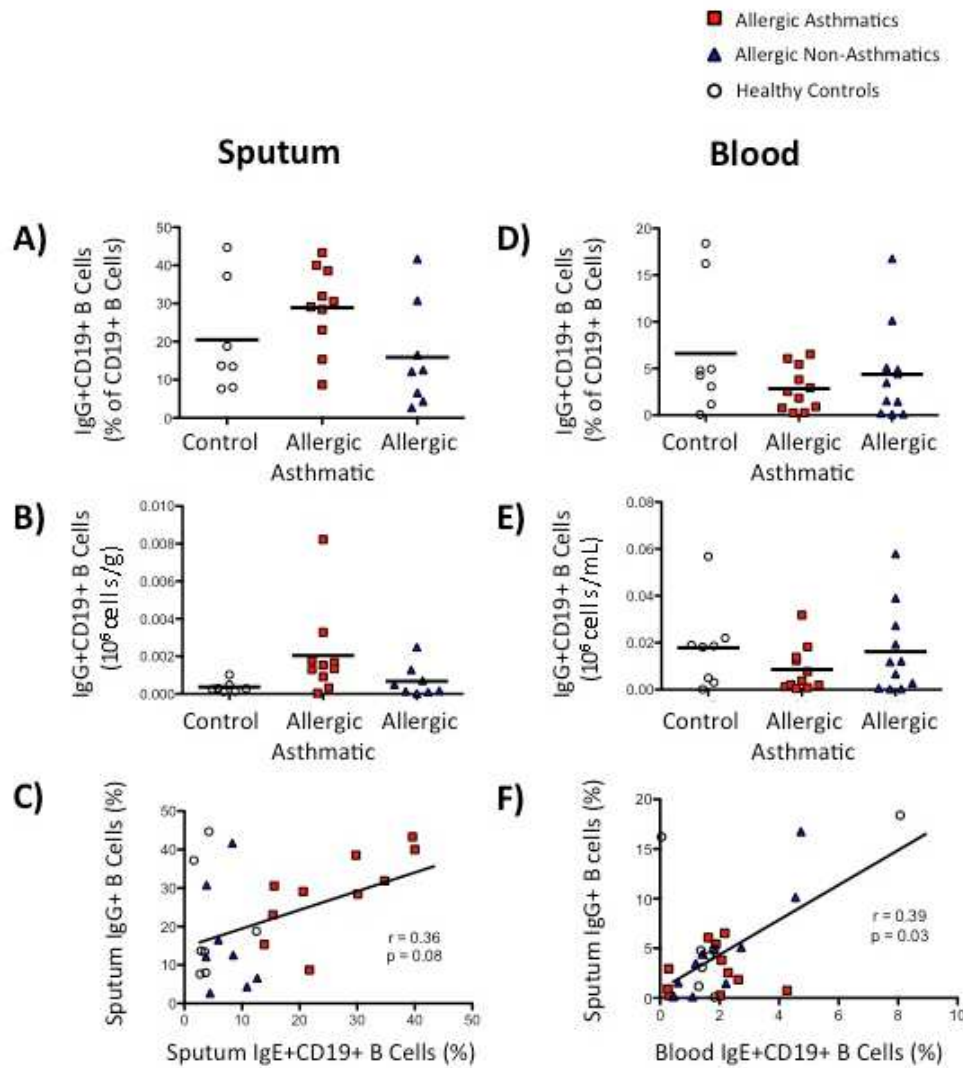
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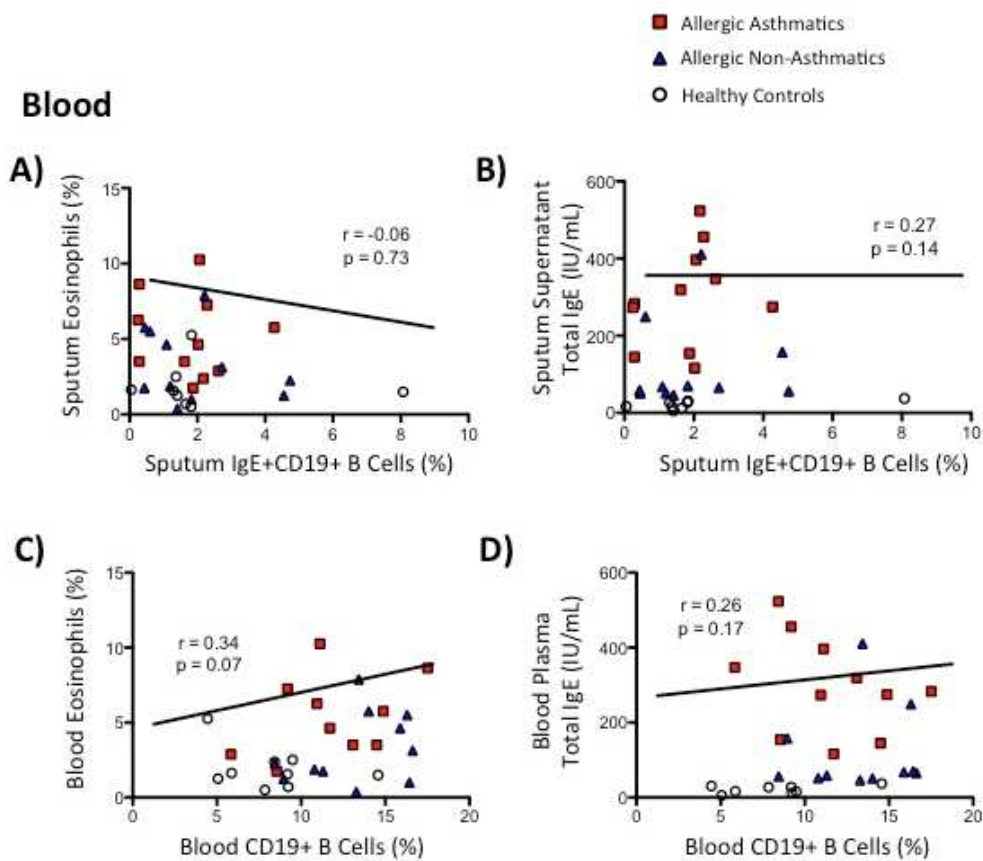
148 **Supplemental Figure E5: The level of lymphocytes in blood and in the sputum of subjects with**
 149 **allergic asthma and non-asthmatic controls.** Data were analyzed using a 1-way ANOVA with Tukey
 150 post-hoc tests and are presented as mean±SEM. In sputum: control n=7, allergic asthmatic n=10,
 151 allergic n=8. In blood: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are
 152 denoted by *, where p<0.05.

153



Supplemental Figure E6: The frequency and numbers of IgG+ B cells and correlation with IgE+ B Cells in the sputum and blood of subjects with allergic asthma and non-asthmatic controls.

Data were analyzed using a 1-way ANOVA with Tukey post-hoc tests and are presented as mean $\pm$ SEM. Correlation data are presented as correlations with Spearman rank r-values and linear regression lines of best fit. In sputum: control n=7, allergic asthmatic n=10, allergic n=8. In blood: control n=11, allergic asthmatic n=11, allergic n=8. Significant findings are denoted by *, where $p < 0.05$.



163

164 **Supplemental Figure E7: The relationship between B cells and IgE+ B cells, and eosinophils and**
 165 **total IgE in blood of subjects with allergic asthma and non-asthmatic controls.** Data are presented
 166 as correlations with Spearman rank r -values and linear regression lines of best fit. In blood: control
 167 $n=11$, allergic asthmatic $n=11$, allergic $n=8$. Exact p -values were denoted, where $p<0.05$ was
 168 considered a significant correlation.