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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Comparison of regulatory B cells in asthma and allergic rhinitis

To the Editor,

B lymphocytes produce antibodies, act as antigen-presenting cells and produce cytokines.¹ They can be classified into functional subsets according to their distinct expression of surface markers and their profile of secreted cytokines. Regulatory B cells (Bregs), control excessive inflammatory responses primarily through secretion of IL-10. IL-10 inhibits proinflammatory cytokine production and supports regulatory T-cell differentiation.²⁻⁴ During the last decade, an essential role has been attributed to IL-10⁺ Bregs in limiting excessive immune reactivity in experimental models of infection, allergic inflammation, autoimmunity, tolerance, tumor growth, and organ transplantation.² An important in vivo functional role for human Bregs has been suggested by the finding that exacerbations of colitis and psoriasis take place after B-cell depletion therapy.⁵ An increase in peripheral CD19⁺CD25⁺CD71⁺CD73⁻ Bregs upon allergen-specific immunotherapy of patients has been linked to the suppression of IgE and upregulation of IgG4 production, as well as increased IL-10 production in allergen-specific T and B cells.³ So far, three different IL-10⁺ Breg subsets have been identified in humans. CD19⁺CD5⁺CD1d⁺ and CD19⁺CD24⁺CD38⁺ Bregs were shown to suppress Th1 cells,^{6,7} whereas CD19⁺CD25⁺CD71⁺CD73⁻ Bregs may play a role in allergen-specific immune tolerance. Moreover, Kamekura et al⁸ have demonstrated significant decrease of CD19⁺CD24^{hi}CD27⁺ B cells in allergic rhinitis and asthma. In contrast, another study reported an increase of CD19⁺CD24^{hi}CD27⁺ B cells and a decline of CD19⁺CD24^{hi}CD38^{hi} B cells in allergic rhinitis compared to healthy individuals.⁹ However, it is noteworthy that none of these studies assessed production of IL-10, which is a crucial feature of Bregs, as a marker of this population. Therefore, in view of this data and the lack of IL-10 measurement in the previous studies, we aimed to compare the percentages of IL-10-producing Breg subsets in peripheral blood from clinically well-characterized asthmatic, allergic rhinitis, and healthy 14-15 years old individuals (Table S1) using multicolor flow cytometry. Peripheral blood mononuclear cells were stained with antibodies specific for IL-10, CD1d, CD5, CD24, CD38, CD25, CD71, and CD73. Three types of Breg cells were studied according to the gating strategy shown in Figure 1A, based on the isotype controls (Figure S1). Since IL-10 has

been proposed as a key suppressive cytokine of Breg cells in vivo and in vitro,²⁻⁴ we aimed to compare the percentage of IL-10-producing B cells between patients with asthma, allergic rhinitis, and healthy controls at day 0. Donor-dependent variation in the total number of IL-10⁺ B cells (ranging between 1% and 24% of total B cells) was observed and there was no difference between the studied groups (Figure 1B). Further comparison of IL-10 expression within the three Breg populations revealed no difference in the percentage of CD19⁺IL-10⁺CD25⁺CD71⁺CD73⁻ (Figure 1C), CD19⁺IL-10⁺CD24⁺CD38⁺ (Figure 1D), and CD19⁺IL-10⁺CD5⁺CD1d⁺ (Figure 1E) Breg cells within or between patients with asthma and allergic rhinitis, and healthy controls at day 0. The comparison of total B cell percentage as well as naïve and memory B cells gated based on the expression of CD27 and IgD (Figure S2A) did not show any significant difference between the studied groups (Figure S2B). Despite the number of samples being limited, this observation may suggest that there is no deficiency in circulating Bregs in asthmatic and allergic rhinitis patients.

As we were interested in the response of B cells to B-cell-specific stimulants, we aimed to analyze if there is any difference in the capacity for the induction of IL-10 expression in the three groups after 3 days of stimulation with CpG oligonucleotides (CpG) or anti-B-cell receptor (α BCR) and CD40 ligand (CD40L). Cells were gated according to the strategy shown in Figure 2A. IL-10 production was significantly induced upon CpG stimulation in patients with asthma and healthy controls. There was a trend toward lower number of IL-10-producing Bregs in patients with allergic rhinitis. Therefore, it would require a larger patient cohort to determine whether patients with allergic rhinitis have a significant Breg deficiency. One can speculate that even the modest differences in Breg frequencies and function, may have an impact on systemic responses, as Bregs have been associated with the suppression of excessive inflammation, restoration of Th1/Th2 balance, inhibition of Th1 and Th17 differentiation, and induction of regulatory T cells, mediated not only through the release of soluble factors, but also via cell-to-cell contact.¹⁰ Moreover, a slight difference in circulating Breg frequencies could reflect a significant difference in tissue-infiltrating Breg cells. Further studies are needed to quantify Breg populations in respiratory mucosal tissues.

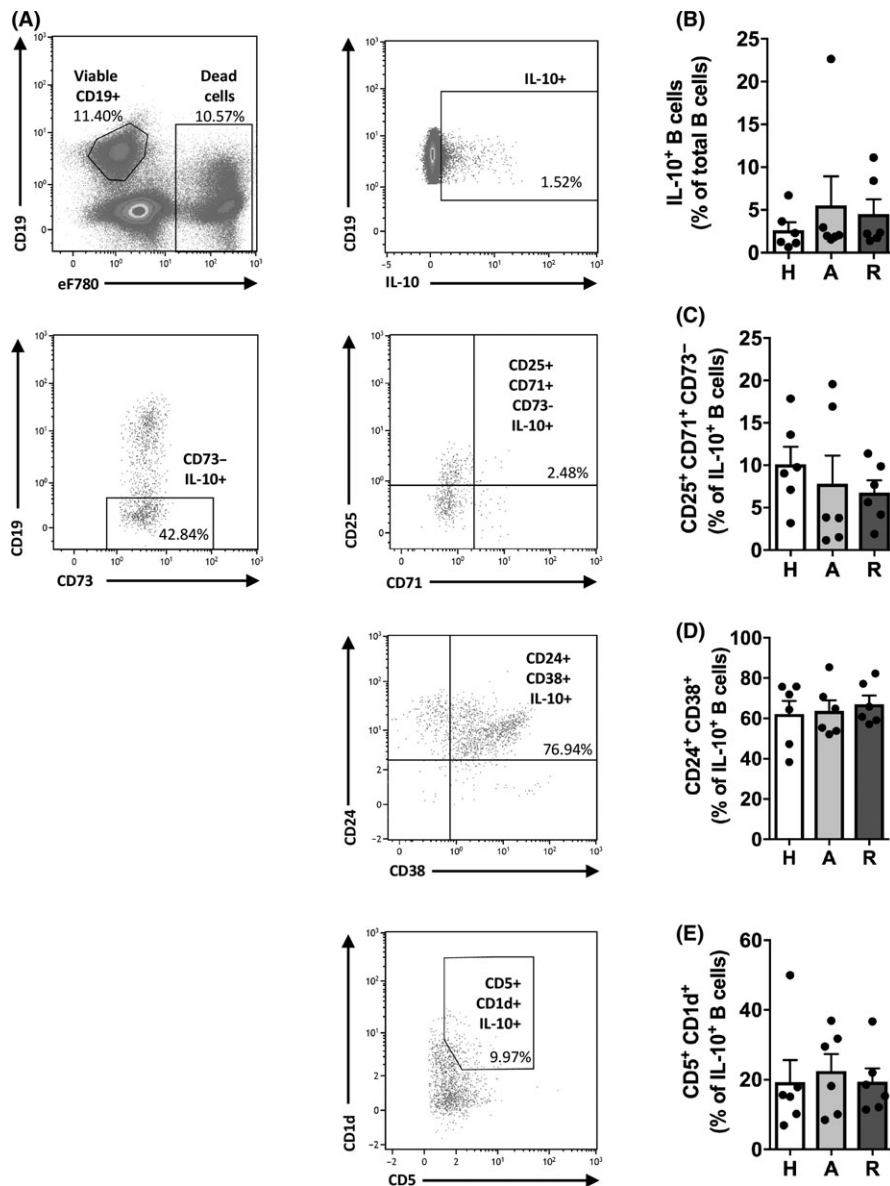


FIGURE 1 Percentages of Breg cell subsets and their expression of IL-10 are similar in patients with asthma (A) or allergic rhinitis (R) compared to healthy individuals (H) at day 0. A, Gating strategy of three different Breg cell subsets in an asthmatic donor. B, Percentage of IL-10⁺ B cells within total B cells. Kruskal-Wallis test was used to compare differences between the groups. C-E, Three different Breg cell subsets: C, CD19⁺CD25⁺CD71⁺CD73⁻; D, CD19⁺CD24⁺CD38⁺; E, CD19⁺CD5⁺CD1d⁺ measured as a percentage of total IL-10⁺ B cells. Wilcoxon matched-pairs signed rank test was used to analyze changes in response to different stimulation. H (n = 6), A (n = 6), R (n = 6). Each bar represents the mean ± SEM

Although, α BCR + CD40L stimulation resulted in the increase of IL-10⁺ B cells in some individuals, there was no significant change (Figure 2B). The patients who responded strongly to α BCR + CD40L did not have less severe symptoms compared to the rest of the subjects in allergic rhinitis group. There was no significant difference in the percentage of viable CD19⁺ B cells in total cells between the studied groups. The CD19⁺CD25⁺CD71⁺CD73⁻ Bregs showed a very profound response to CpG and α BCR + CD40L stimulations in patients with asthma, whereas in patients with allergic rhinitis this increase was observed only after CpG treatment (Figure 2C). However, a significant induction of CD19⁺CD25⁺CD71⁺CD73⁻ Bregs was influenced by donor-dependent variation in response to CpG stimulation. The other two subsets, CD19⁺CD24⁺CD38⁺ Bregs (Figure 2D) and CD19⁺CD5⁺CD1d⁺ (Figure 2E) were decreased upon stimulation with both CpG and α BCR + CD40L. In line with a previous study, we observed an increase in IL-10⁺ B cells upon stimulation with CpG³ and α BCR + CD40L.⁷ Although CpG is a potent

stimulus for inducing IL-10 production by B cells, there was no significant difference between the groups. The observed decrease in the percentage of CD19⁺CD24⁺CD38⁺ Bregs upon stimulation with both CpG and α BCR + CD40L may be explained by the fact that CD19⁺CD24⁺CD38⁺ Bregs develop from transitional B cells and they might have already differentiated after three days of stimulation into a less CpG responsive B-cell population. Interestingly, we found that CD24⁺CD38⁺ Breg cells express surface markers associated with other described Breg subsets. Most prominently, CD24⁺CD38⁺ Breg cells also express CD71 and CD5 (data not shown).

In conclusion, our results showed no differences in numbers of peripheral Bregs both at baseline and after stimulation in patients with asthma and allergic rhinitis compared to healthy controls. Although we observed a trend toward decreased induction of IL-10-producing B cells in response to stimulation in patients with allergic rhinitis, due to limited number of study subjects, we

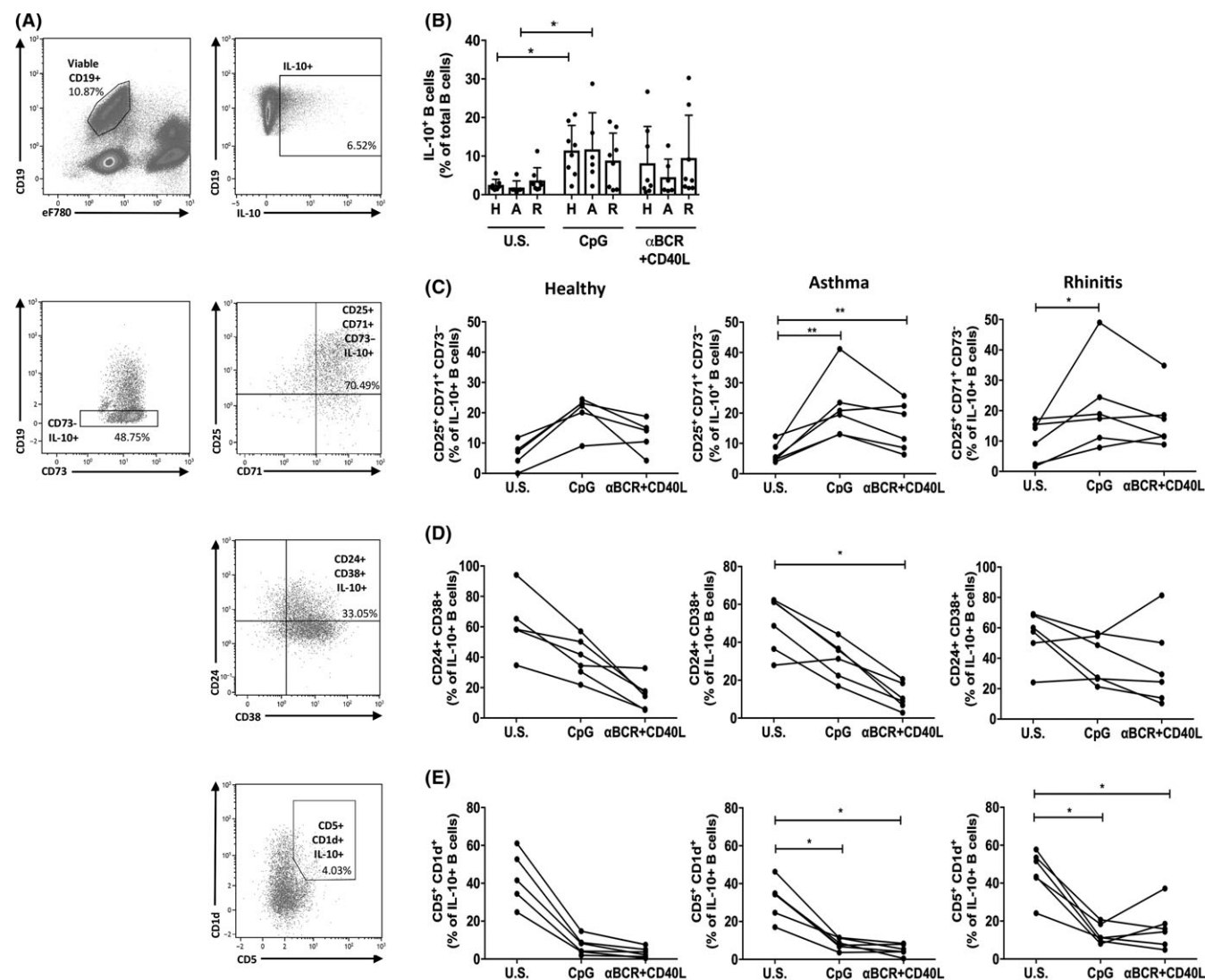


FIGURE 2 Three Breg cell subsets are responding differently to CpG and αBCR + CD40L stimulations compared to unstimulated control (U.S.) in patients with asthma (A) or allergic rhinitis (R) and healthy individuals (H) at day 3. A, Gating strategy of three different Breg cell subsets shown in an asthmatic donor. B, Increase in IL-10 expression in total B cells. (n = 8), A (n = 6), R (n = 8). C, D, Three different Bregs subsets: C, CD19⁺CD25⁺CD71⁺CD73⁺; D, CD19⁺CD24⁺CD38⁺; E, CD19⁺CD5⁺CD1d⁺ measured as a percentage of total IL-10⁺ B cells. Wilcoxon matched-pairs signed rank test was used to analyze changes in response to different stimulation. *P < 0.05; **P < 0.01. H (n = 5-6), A (n = 6), R (n = 6). Each bar represents the mean ± SEM

cannot conclude whether there is a deficiency. It is likely that we did not observe an impaired induction of Bregs in response to stimulation as both patients with asthma and allergic rhinitis were under treatment. Nonetheless we cannot exclude that exacerbation of asthma or allergic rhinitis may influence the Breg responses. It is likely that frequency of Bregs in peripheral blood may not reflect the potential impairment in local regulation of Bregs. Direct responses should be studied in the nasal tissue and induced sputum. It has been demonstrated that IgE⁺ B cells were existing in sputum but not in blood and bone marrow. Further research on a larger population focusing on IL-10-producing Bregs in the periphery and affected organs may be necessary to conclude whether there is a deficiency in Breg subsets in patients with allergic rhinitis and asthma.

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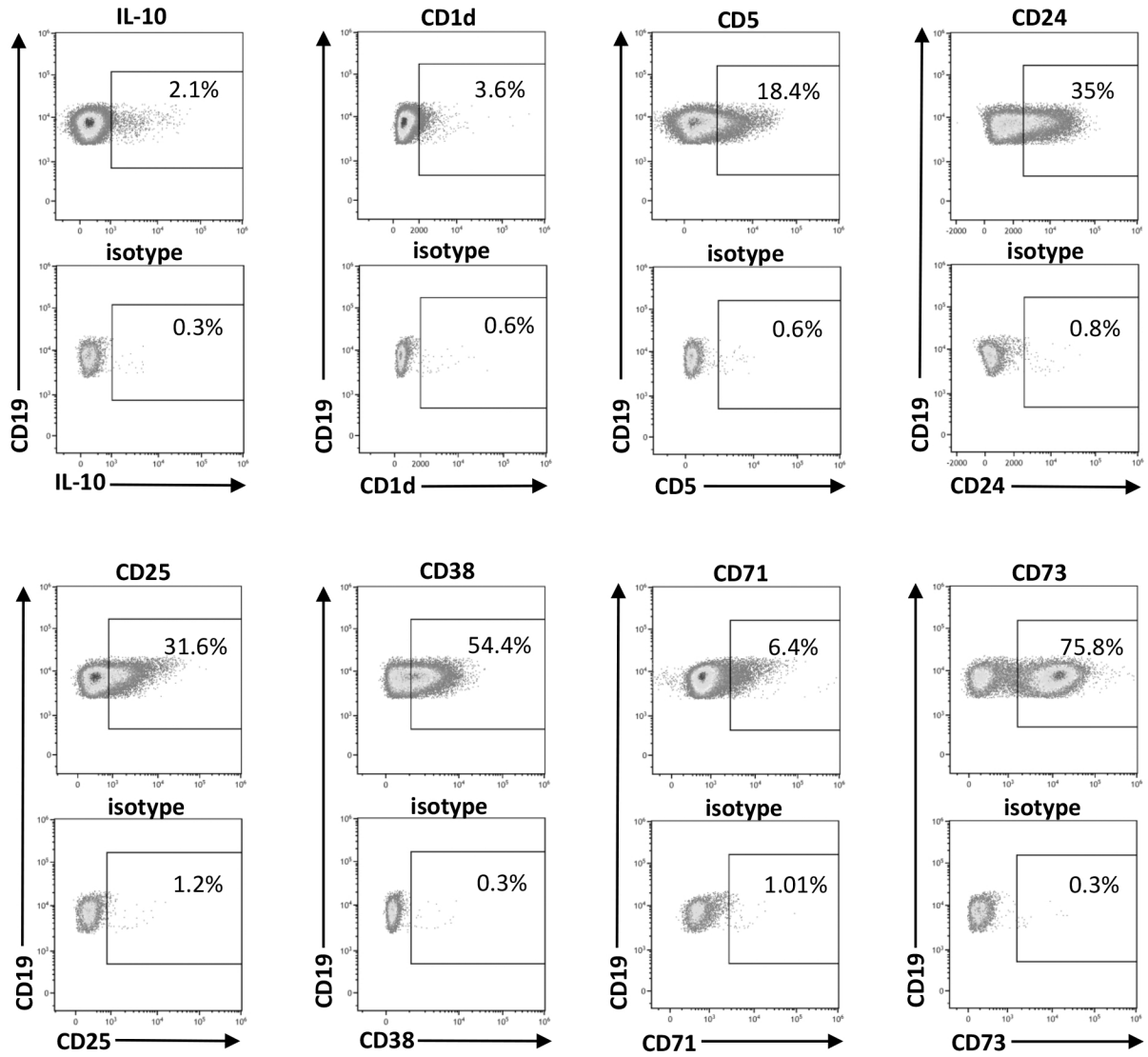
Wirz and Głobińska equally contributed to this study.

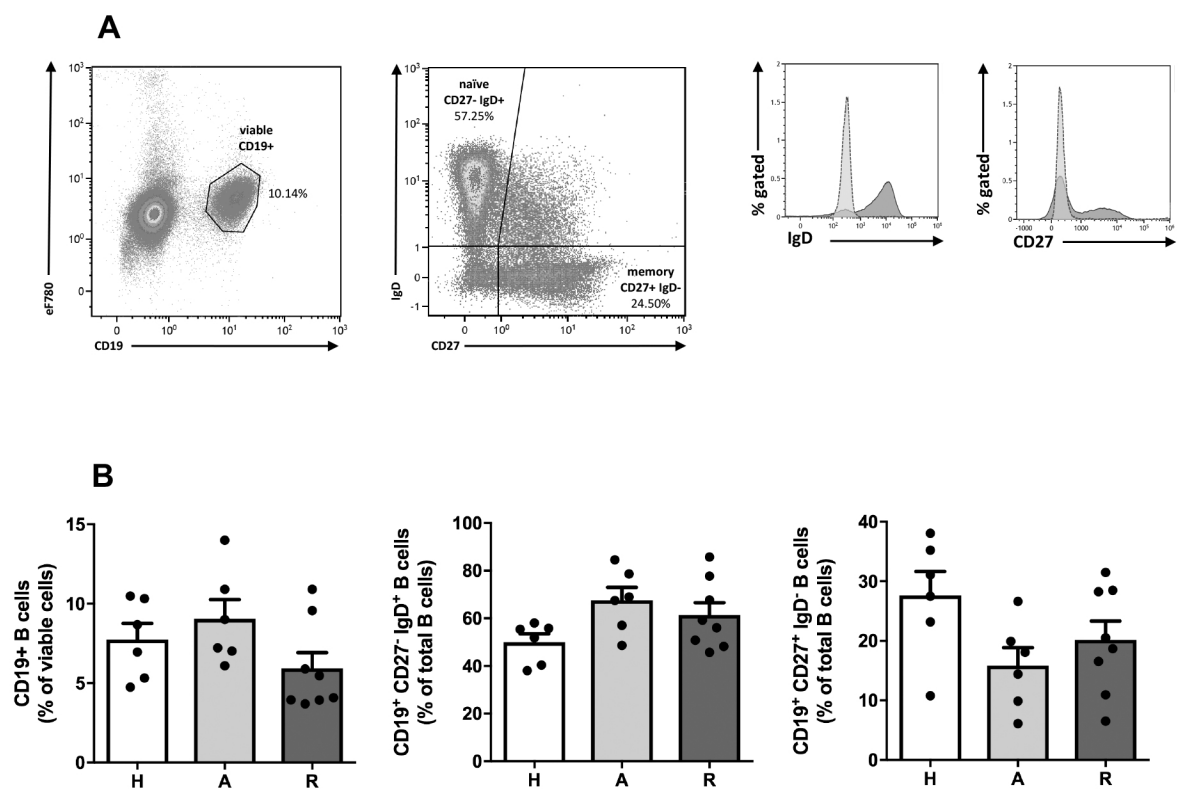
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Supporting information

Letter to the Editor

Comparison of regulatory B cells in asthma and allergic rhinitis

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3 **Participants**

4 The study participants were enrolled
5 within the MeDALL (Mechanisms
6 of the Development of ALLergy) research
7 collaboration supported by the European
8 Union under the Health Cooperation
9 Work Programme of the 7th Framework
10 programme (grant agreement number
11 261357). The study included 14-15 years
12 old atopic subjects with asthma (n=6) or
13 rhinitis (n=8), all diagnosed at 14 years
14 and/or 6 years follow-up at the DARC
15 (Danish Allergy Research Center) cohort
16 and healthy, non-atopic individuals (n=8).
17 Atopy was defined as skin prick tests
18 (SPT) $\geq$ 3mm and/or s-IgE $>$ 0,35 kU/l.
19 Rhinitis was diagnosed with $\geq$ 2 episodes
20 either sneezing, runny/blocked nose
21 and/or itchy, red and watery eyes apart
22 from common cold. Asthma was
23 diagnosed as either recurrent cough,
24 wheeze, and/or shortness of breath not
25 triggered by infection. Additionally,
26 reversibility test with β 2-agonist was
27 performed in patients with asthma.¹ The
28 study has been approved by the
29 Regional Scientific Ethical Committee for
30 Southern Denmark (S-VF-19980101).

31

32 **Peripheral blood mononuclear cells** 33 **(PBMCs) isolation**

34 Blood samples from all study subjects
35 were drawn after obtaining informed
36 consent. Citrate-phosphate-adenine
37 (CPDA-1) was used as anticoagulant
38 preservative. PBMCs were isolated using
39 Ficoll-Hypaque (Sigma, St. Louis, MO,
40 USA) density-gradient centrifugation.
41 Density gradient-isolated PBMC were
42 resuspended in ice-cold fetal bovine
43 serum with 10% dimethyl sulfoxide
44 (DMSO) at 10^7 cells/ml. Aliquots of cell
45 suspension were transferred to cryovials,
46 cryopreserved using a controlled-rate

47 freezing apparatus, and stored at -196°C
48 until analysis.

49 After thawing, cells were resuspended in
50 cRPMI medium and left overnight
51 in 37°C , 5% CO_2 . The next day, cells
52 were counted. One quarter of the cells
53 was used for day 0 measurement, and
54 the remaining cells – for 3 days
55 stimulation.

56 At day 0 cells were stimulated for 2h with
57 25 ng/ml phorbol 12-myristate 13-
58 acetate, PMA (Sigma-Aldrich Chemie
59 GmbH, Buchs, Switzerland) and 1 $\mu\text{g/ml}$
60 ionomycin (Sigma-Aldrich Chemie
61 GmbH, Buchs, Switzerland) and then for
62 another 2h with 10 $\mu\text{g/ml}$ Brefeldin A
63 (Sigma-Aldrich Chemie GmbH, Buchs,
64 Switzerland). In parallel, cells were
65 stimulated with 1 μM CpG ODN 2006
66 (MicroSynth, Balgach, Switzerland),
67 5 $\mu\text{g/ml}$ anti-BCR (Jackson
68 Immunoresearch Europe, Newmarket
69 Suffolk, UK) and CD40L-expressing
70 mouse fibroblasts (5×10^4 cells/well in a
71 24 well plate) or left unstimulated (U.S.).
72 At day 3, cells were further stimulated
73 with PMA/Ionomycin/Brefeldin A as
74 described above. All the samples from
75 day 0 and day 3 were harvested and
76 used for the flow cytometry staining.

77

78 **Flow cytometry**

79 After harvesting, cells were washed with
80 PBS and incubated with the viability dye
81 eF780 (eBioscience, Frankfurt,
82 Germany) for 30 min at 4°C , then
83 washed and stained with the antibodies
84 for the surface markers for 20 min at 4°C
85 in the dark. After staining, samples were
86 directly measured or stained for
87 intracellular cytokines using the
88 Cytofix/Cytoperm (BD Biosciences,
89 Allschwill, Switzerland) according to
90 manufacturer's protocol. The antibodies
91 used for this study are listed in Tables

E2-3. Data were acquired on Gallios (Beckman Coulter, Brea, CA) and analyzed with Kaluza Software (Beckman Coulter, Brea, CA).

96

97 **Statistical analysis**

98 Statistical analysis was performed using
99 GraphPad Prism version 7 (GraphPad
100 Software, Inc, La Jolla, CA). Differences
101 between the studied groups were tested
102 for normality of data and analyzed with
103 Kruskal-Wallis test. Changes in response
104 to different stimulations were analysed
105 using Wilcoxon matched-pairs signed
106 rank test. Data were considered
107 significantly different when $p < 0.05$.

108

109 **ONLINE TABLES**

110 **Table S1.** Patient characteristics.

111

		healthy [H]	asthma [A]	allergic rhinitis [R]
Positive SPT	Number	8	6	8
	Perennial allergens only	0	4	2
	Seasonal allergens only	0	2	3
	Perennial and seasonal allergens	0	0	3
Sex (M/F)		3/5	4/2	5/3
Age		14-15		
Total IgE [IU/ml]		25.48 ± 21.24	261.18 ± 286.78	712.88 ± 1426.87
Asthma parameters	FEV ₁	NA	3.22 ± 0.76	NA
	FEV ₁ /FVC %	NA	78.90 ± 9.68	NA
	Treatment	Local steroid	NA	4
		β ₂ -agonist	NA	3
	Treatment	Antihistamines	NA	NA
		Nasal steroid	NA	7

112

113

114

115 **Table S2.** B cell developmental stages.

Marker	Label	Clone	Company
IgD	FITC	IA6-2	BD Biosciences
CD27	PE	M-T271	BD Biosciences
CD10	PE-CF594	HI10a	BD Biosciences
IgM	PerCP/Cy5.5	MPC-11	Biolegend
CD38	PE-Cy7	HIT2	Biolegend
CD138	AF647	MI-15	Biolegend
CD20	AF700	2H7	Biolegend
CD24	Brilliant Violet 421	ML5	Biolegend
CD19	Brilliant Violet 510	HIB19	Biolegend
Isotype control	Label	Clone	Company
mlgG2a, k	FITC	G155-178	BD Biosciences
mlgG1, k	PE	MOPC-21	BD Biosciences
mlgG1, k	PE-CF594	X40	BD Biosciences
mlgG1, k	PerCP/Cy5.5	MOPC-21	Biolegend
mlgG1, k	PE-Cy7	MOPC-21	Biolegend
mlgG1, k	AF647	MOPC-21	Biolegend
mlgG2b, k	AF700	MPC-11	Biolegend
mlgG2a, k	Brilliant Violet 421	MOPC-173	Biolegend

116

117

118 **Table S3.** Regulatory B cells.

Marker	Label	Clone	Company
CD71	FITC	CY1G4	Biolegend
CD1d	PE	51.1	eBiosciences
CD25	PE-CF594	M-A251	BD Biosciences
CD38	PerCP/Cy5.5	HIT2	Biolegend
IL-10	PE-Cy7	UCHT2	Biolegend
CD73	APC	AD2	Biolegend
CD5	AF700	CRIS-1	Novusbio
CD24	Brilliant Violet 421	ML5	Biolegend
CD19	Brilliant Violet 510	HIB19	Biolegend
Isotype control	Label	Clone	Company
mlgG2a, k	FITC	RTK2758	Biolegend
mlgG2b, k	PE	eBMG2b	eBiosciences
mlgG1, k	PE-CF594	X40	BD Biosciences
mlgG1, k	PerCP/Cy5.5	MOPC-21	Biolegend
rlgG1, k	PE-Cy7	MOPC-21	Biolegend
mlgG1, k	APC	MOPC-21	Biolegend
mlgG2a, k	AF700	MG2a-53	Novusbio
mlgG2a, k	Brilliant Violet 421	MOPC-173	Biolegend

119

120

FIGURE LEGEND

Figure S1. Flow cytometry measurements showing specific staining and corresponding isotype controls for markers used to identify different Breg subsets. Staining performed at day 3, gated on viable B cells, unstimulated.

Figure S2. Percentages of B cells are similar in asthmatic (A), allergic rhinitis (R) and healthy donors (H).

A. Gating strategy for total CD19⁺ B cells, CD19⁺CD27⁺IgD⁺ B cells (naïve) and CD19⁺CD27⁺IgD⁻ B cells (memory). Overlap histograms showing specific stain and corresponding isotype controls for IgD and CD27. Gated on viable B cells. Dark histogram – specific stain, light histogram – isotype control.

B. Percentage of CD19⁺ B cells within viable cells and CD19⁺CD27⁺IgD⁺ B cells (naïve), and CD19⁺CD27⁺IgD⁻ (memory) B cells in total B cells.—Kruskal-Wallis test was used to compare differences between the groups. H (n=6), A (n=6), R (n=8). Each bar represents the mean ± SEM.

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