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John-Paul Oliveria, PhD, Amani I. El-Gammal, MD, Michelle Yee, BSc, Caitlin D. Obminski, BSc, Tara X. Scime, BSc, Rick M. Watson, BSc, Karen Howie, BSc, Paul M. O'Byrne, MD, Roma Sehmi, PhD, Gail M. Gauvreau, PhD

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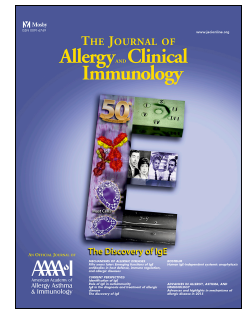
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Letter to the Editor

Changes in regulatory B cell levels in bone marrow, blood and sputum of asthmatics following inhaled allergen challenge

John-Paul Oliveria, PhD, Amani I. El-Gammal, MD, Michelle Yee, BSc, Caitlin D. Obminski, BSc, Tara X. Scime, BSc, Rick M. Watson, BSc, Karen Howie, BSc, Paul M. O'Byrne, MD, Roma Sehmi, PhD, Gail M. Gauvreau, PhD*

¹Department of Medicine, Division of Respiriology, McMaster University, Hamilton, Ontario

***Corresponding Author**

Gail M Gauvreau PhD

Professor, Department of Medicine, Division of Respiriology

McMaster University, HSC 3U26, 1200 Main St West, Hamilton, ON Canada

Telephone: 905-525-9140 x22791

Fax: 905-528-1807

E-mail: gauvreau@mcmaster.ca

CAPSULE SUMMARY: Following allergen exposure in the airways of subjects with allergic asthma, B_{regs} appear to efflux from the bone marrow and accumulate in the lungs with a possible role in dampening local inflammatory responses.

KEY WORDS: allergy, asthma, B cells, regulatory B cells, sputum, blood, bone marrow

LIST OF ABBREVIATIONS

B_{regs} - regulatory B cells

CD - cluster of differentiation

FEV₁ - forced expiratory volume in 1 second

FoxP3 - forkhead box p3

Ig - immunoglobulin

IL - interleukin

MCh PC₂₀ - provocative concentration of methacholine inducing at least a 20% fall in FEV₁

T_{regs} - regulatory T cells

To the Editor:

Aeroallergens inhaled into allergic asthmatic airways initiate type 2 inflammation, including production of the cytokines IL-4 and IL-13 known to promote maturation of IgE-producing B-cells^{1,2}. A novel subset of B-cells, termed regulatory B cells (B_{regs}) have been proposed to suppress IgE-mediated allergic inflammation via secretion of IL-10³⁻⁶; whereby a deficiency in B_{regs} has been associated with increased serum IgE, increased type 2 cytokines, and increased eosinophilia in murine models of allergic airway disease^{3,7}. However, few human studies have evaluated the role B_{regs} play in human models of asthma^{8,9}. We hypothesize that B_{regs} are dysregulated in allergic asthmatics compared to controls and that the levels of B_{regs} are affected by type 2 mediated allergic inflammation. This study (i) compared phenotypes of B_{regs} across subjects with allergic asthma, and non-asthmatics with or without allergies, (ii) determined the kinetics and relative compartmental distribution (hematopoietic, systemic and local airway compartments) of B_{regs} in allergic asthmatics following allergen inhalation challenge, and (iii) investigated the functional capacity B_{regs} to express IL-10 *in vitro*.

We recruited eleven allergic asthmatics, seven allergic non-asthmatics, and seven healthy controls (Table EI), all non-smokers and between 18 and 65 years of age. All subjects provided informed consent and underwent screening to determine allergic status by skin-prick-test, and lung function by spirometry. No subjects required medication for treatment of asthma or allergies. Asthmatics had a forced expiratory volume in one second (FEV₁) ≥70% of predicted, methacholine provocative concentration causing at least a 20% fall in FEV₁ (PC₂₀) ≤16 mg/ml, and were excluded if they had exacerbations or respiratory tract infections within four weeks, or underlying diseases other than asthma. All eligible subjects in the three groups donated a blood sample for the enumeration of B_{regs}, which are identified as (1) CD5+, (2) CD1d+CD5+, (3) CD24+CD27+, and (4) CD24+CD38+. Ten additional subjects (Table EII) with allergic asthma provided samples of sputum, blood and bone marrow on day 1, followed by randomization to allergen or diluent inhalation challenge on day 2. Spirometry was measured up to 7-hours post-challenge when sputum and blood samples were

collected. On day 3 (24-hours post-challenge), methacholine challenge, sputum, blood and bone marrow collection were performed. This triad was then repeated with the other inhaled agent (allergen or diluent) after a 4-week recovery period. Lastly, six additional subjects (Table EIII) with allergic asthma provided blood samples to isolate CD19⁺ B cell and perform *in vitro* cultures with IL-4.

Sputum cells were surface-stained with antibodies to CD45, CD19, and intracellularly with FoxP3 to enumerate B_{regs}. Peripheral blood and bone marrow mononuclear cells were surface-stained with antibodies to CD19, CD1d, CD5, CD24, CD27, CD38, and intracellularly with IgE, IL-10, and FoxP3 to enumerate four different B_{regs} phenotypes, particularly expressing FoxP3 (CD5+FoxP3+, CD1d+CD5+FoxP3+, CD24+CD27+FoxP3+, CD24+CD38+FoxP3+). Appropriate isotypes were used as negative controls. Cells were acquired using a Becton Dickinson LSRFortessa flow cytometer (BD Bioscience, San Jose, California, United States), and the files were analyzed with the FlowJo software (TreeStar, Ashland, Oregon, United States). The subject criteria, sample processing, cell-staining procedures, and gating strategies are detailed in the Supplementary Methods and Fig E1. All data are presented as the mean $\pm$ SEM.

In blood, the frequency of CD19⁺ B-cells expressed as a percentage of all lymphocytes was similar between groups (Fig 1A). However, the frequency of CD19+FoxP3⁺ B-cells expressed as a percentage of total CD19⁺ B-cells was approximately 2-fold lower in asthmatic subjects ($6.1 \pm 0.4\%$) compared to allergic subjects ($11.6 \pm 0.9\%$) and controls ($12.8 \pm 1.3\%$) ($P < 0.05$, Fig 1B). The frequency of CD5⁺ B_{regs} and CD24+CD27⁺ B_{regs} were also lower in asthmatic subjects compared to controls ($P < 0.05$, Fig 1C&E). When co-expression of FoxP3 was examined, the frequency of CD5+FoxP3⁺ B_{regs} and CD24+CD38+FoxP3⁺ B_{regs} were both lower in asthmatic subjects compared to controls ($P < 0.05$, Fig 1C&F). In contrast, the frequency of CD1d+CD5⁺ and CD1d+CD5+FoxP3⁺ B_{regs} was not different across groups (Fig 1D).

After allergen challenge, the frequency of CD19⁺ B-cells expressed as a percentage of lymphocytes did not change in blood or bone marrow compartments, but increased in sputum at 7-

hours post-allergen challenge, compared to diluent (Fig E2A-C, Table EIV). The frequency of CD19+FoxP3+ B-cells expressed as a percentage of CD19+ B-cells, decreased in bone marrow at 24-hours post-allergen challenge, but increased in blood at 7-hours and sputum at 24-hours post-allergen challenge, compared to diluent ($P<0.05$, Fig 2A, Table EIV).

In bone marrow, there were no allergen-induced changes in any of the B_{reg} phenotypes compared to diluent challenge. In blood, there were significantly lower levels of CD5+ B_{regs} and CD1d+CD5+ B_{regs} at 7-hours post-allergen challenge, while there were significantly higher levels of CD24+CD38+ B_{regs} at 24-hours post-allergen challenge, compared to diluent ($P<0.05$, Fig E2D-E Table EV). Furthermore, compared to diluent, allergen consistently decreased the percentage of FoxP3 positive B_{reg} phenotypes in the bone marrow ($P<0.05$, Fig 2B, Fig E2D, Table EV) and increased the percentage of FoxP3 positive B_{reg} phenotypes in blood ($P<0.05$, Fig 2B, FigE2E, Table EV) at 24-hours post-allergen challenge.

CD19+ B-cells purified from blood of allergic asthmatics and incubated with 5 ng/ml and 25 ng/mL of IL-4 for 72 hours significantly increased expression of FoxP3 and co-expression of FoxP3 and IL-10 ($P<0.05$, Fig 2C). In addition, IL-4 induced significantly higher proportions of CD5+FoxP3+IL-10+ and CD24+CD27+IL-10+ B_{regs} compared to control ($P<0.05$, Fig 2C, Fig E3). Murine models suggests that B_{regs} have a role in dampening allergic immune responses through IL-10 dependent mechanisms^{4-6,8}, and further studies are required to confirm this in human models.

In summary, we evaluated phenotypes of B_{regs} implicated in allergic disease^{4,8,9} and found lower circulating levels of B_{regs} in allergic asthmatics compared to controls, suggesting allergic immune processes. We observed a consistent decrease in the frequency of FoxP3-expressing B_{regs} in bone marrow and increase in blood at 24-hours post-allergen. Although we were not able to measure B_{regs} in the airways due to limited sample availability, we observed increased frequency of CD19+FoxP3+ B-cells in sputum. The observed compartmental differences in the levels of CD19+FoxP3+ B-cells after allergen challenge suggests the mobilization and homing of B_{regs} from bone marrow, trafficking

through the blood, and into the airways. IL-4 stimulation of B cells induced higher proportions of IL-10-expressing B_{regs} indicating that type 2 cytokine exposure may direct certain B-cell subsets towards a regulatory role to dampen the allergic immune response. Taken together, induction of airway inflammation might be partially due to lower levels of B_{regs}, while the persistence of inflammation in allergic asthma may be due to impaired local regulation by B_{regs}, and thus may be important in severe refractory asthmatics who have uncontrolled chronic lung inflammation. Future therapeutics enhancing the functional role of B_{regs} may be warranted. In conclusion, our data support a role of B_{regs} to dampen the allergic immune response, however, a consensus of how to phenotypically define B_{regs} would be helpful in delving further into the functional capacity of these cells.

John-Paul Oliveria, BSc,
Amani I. El-Gammal, MD,
Michelle Yee, BSc,
Caitlin D. Obminski, BSc,
Tara X. Scime, BSc,
Rick M. Watson, BSc,
Karen Howie, BSc,
Paul M. O'Byrne,
Roma Sehmi, PhD,
Gail M. Gauvreau, PhD

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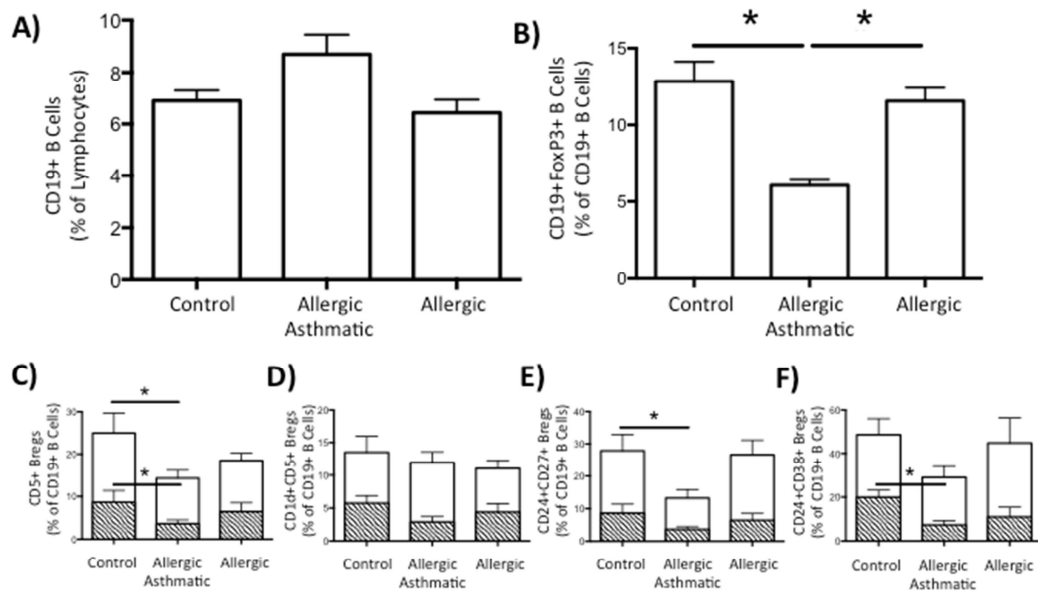
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157 of circulating type 2 follicular helper T cells and regulatory B cells underlies the comorbid
158 association of allergic rhinitis with bronchial asthma. Clin Immunol 2015;158:204–11.
159

FIGURE CAPTIONS

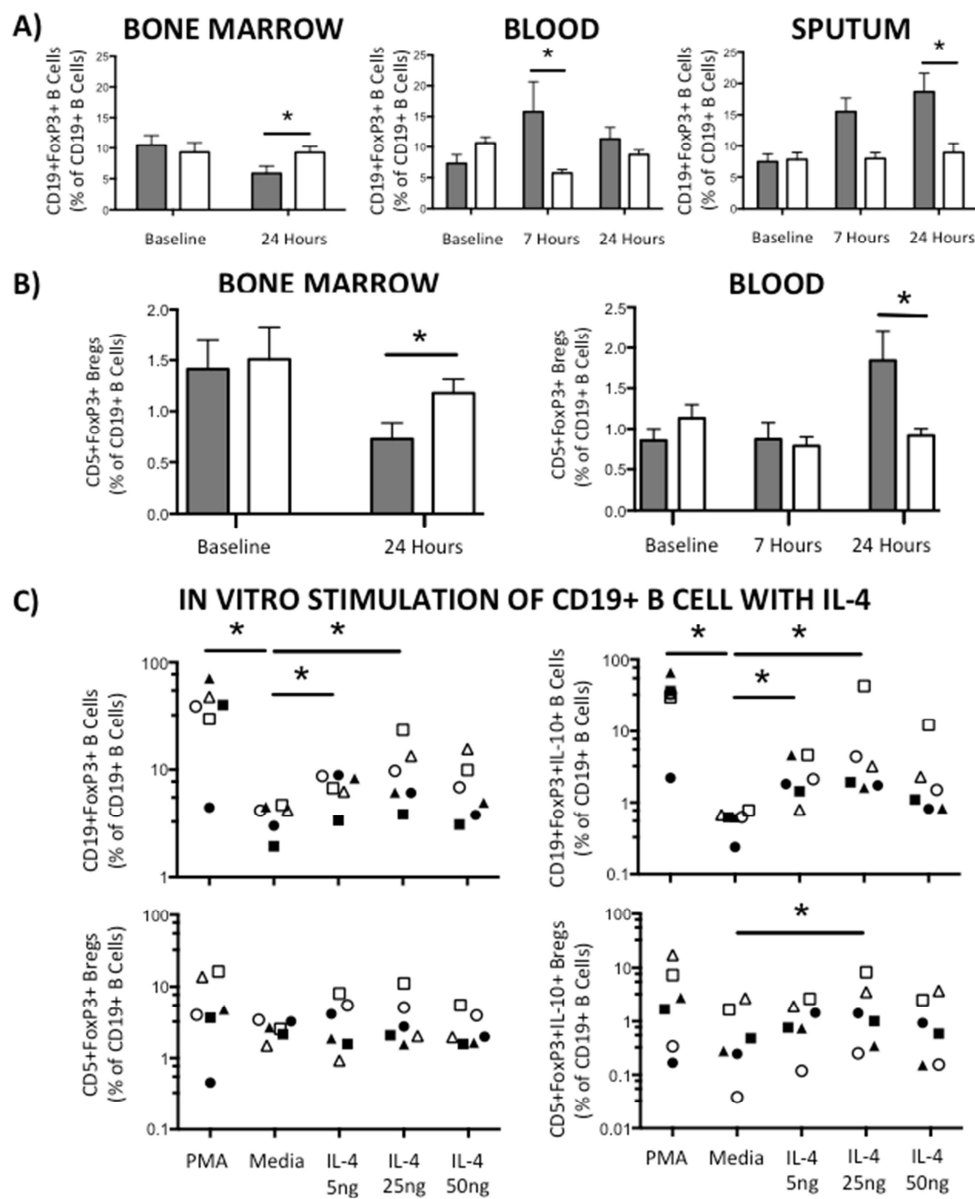
Figure 1: The blood levels of (A) CD19⁺ B cells, (B) CD19⁺FoxP3⁺ B cells, and (C-F) regulatory B cell phenotypes (open bars) and proportions of regulatory B cells expressing FoxP3 (hatched bars) in allergic asthmatics (n=11), allergic non-asthmatics (n=7) and healthy controls (n=7).

Figure 2: (A) The frequency of CD19⁺FoxP3⁺ B cells expressed as a percentage of CD19⁺ B cells in bone marrow (n=10), blood (n=10), and sputum (n=8) of allergic asthmatic subjects at baseline, 7 hours and 24 hours after allergen (grey bars) and diluent (white bars) inhalation challenge. (B) The frequency of CD5⁺FoxP3⁺ B_{regs} expressed as a percentage of CD19⁺ B cells in bone marrow (n=10) of allergic asthmatic subjects at baseline and 24 hours after inhalation challenge and blood (n=10) of allergic asthmatic subjects at baseline, 7 hours and 24 hours after inhalation challenge. (C) IL-4-induced intracellular expression of FoxP3 and IL-10 of CD19⁺ B cells and CD5⁺ B_{regs} in CD19⁺ B cells purified from blood of allergic asthmatic subjects, where each symbol represents a single subject (n=6).

BLOOD



■ Allergen Challenge
□ Diluent Challenge



Supplementary Material**Changes in regulatory B cell levels in bone marrow, blood and sputum of asthmatics following inhaled allergen challenge**

John-Paul Oliveria, PhD, Amani I. El-Gammal, MD, Michelle Yee, BSc, Caitlin D. Obminski, BSc, Tara X. Scime, BSc, Rick M. Watson, BSc, Karen Howie, BSc, Paul M. O'Byrne, MD, Roma Sehmi, PhD, Gail M. Gauvreau, PhD*

Department of Medicine, Division of Respiriology, McMaster University, Hamilton, Ontario

***Corresponding Author**

Gail M Gauvreau PhD

Professor, Department of Medicine, Division of Respiriology

McMaster University, HSC 3U26, 1200 Main St West, Hamilton, ON Canada

Telephone: 905-525-9140 x22791

Fax: 905-528-1807

E-mail: gauvreau@mcmaster.ca

SUPPLEMENTARY METHODS

Clinical Tests

Spirometry, Skin Prick Test, Methacholine Challenge: Spirometry was performed following ATS guidelines². Skin prick tests were performed with common aeroallergen extracts and histamine and saline were used as positive and negative controls, respectively, to confirm atopic status and determine a suitable extract for inhalation³⁻⁵. A methacholine challenge test was completed using the tidal breathing method from a Wright Nebulizer to determine the provocative concentration that resulted in a 20% fall in FEV₁ (PC₂₀)⁶.

Allergen and Diluent Challenges: The methacholine PC₂₀ and skin prick test were used to determine the allergen dose for inhalation, as previously described⁷. Increasing allergen concentrations were inhaled during tidal breathing for 2 minutes, and FEV₁ was measured 10 minutes after each dose. Allergen inhalation was stopped when the FEV₁ fell by at least 20%, then FEV₁ was measured regularly until 7 hours post-challenge. The diluent control challenge consisted of three 2-minute inhalations of the diluting solution (0.9% saline).

Sample Collection and Cell Processing

Sputum Cells: Sputum was induced by inhalation of 3, 4 and 5% saline and mucous plugs were selected and processed as previously described^{8,9}. Briefly, sputum was treated with Dulbecco's phosphate buffered saline (DPBS) and dithiothreitol (DTT) and filtered through a nylon mesh, the cells were pelleted by centrifugation¹⁰. Two cytospins were prepared and stained with Diff Quik for cell differentials and the remaining cells were stored at 4°C for flow cytometry staining.

Peripheral Blood and Bone Marrow Mononuclear Cells: Peripheral blood was collected into vacutainers containing heparin. A total cell count was performed using a hemocytometer. Bone marrow was sampled from the iliac crest following local administration of lidocaine, and collected into a bone marrow aspiration needle containing 1 milliliter of heparin. The blood and bone marrow samples were diluted with two times the volume of 10% fetal bovine serum (FBS) in Roswell Park Memorial

Institute (RPMI) buffer, layered on AccuPrep density gradient, and then centrifuged at 800g for 20 minutes. The peripheral blood mononuclear cell (PBMC) and bone marrow mononuclear cell (BMMC) layers were collected and stored at 4°C for flow cytometry staining.

B Cell Isolation and Culture

A commercially available kit was used to isolate CD19⁺ B cells from whole blood (STEMCELL Technologies Inc., BC, CAN). Briefly, whole blood was mixed gently and incubated for 5 minutes with magnetic beads and B cell isolation cocktail in a 50mL conical tube. The solution was washed with PBS and placed in a magnet to incubate for 5 minutes. The clear fraction suspension above a red whole blood cell layer was pipetted into a new 50mL conical tube. This isolation process was repeated 3 times to isolate the B cells, which were suspended at a concentration of 1×10^6 B cells per millilitre of sterile RPMI media supplemented with 10% FBS, 2.5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.5% Penicillin/Streptomycin, and 1% sodium pyruvate. Using flow cytometry, CD19⁺ B cells were enumerated for purity, and the CD19⁺ B cell isolation yielded $98.8 \pm 0.1\%$ CD19⁺ B cells.

Isolated B cells were plated in a round-bottom 96-well plate at a starting density of 2×10^5 cells per millilitre. The B cells were cultured in the presence or absence of IL-4 at three concentrations (5 ng/mL, 25 ng/mL and 50 ng/mL) (R&D Systems, MN, USA). Additionally, a media-alone (negative control) and phorbol 12-myristate 13-acetate (PMA)-alone (positive control) condition were included. (Invitrogen, CA, USA). B cells were incubated in a humidified 37 degrees Celsius, 5% carbon dioxide incubator for 72 hours. Six hours before the B cells were taken out of culture, a protein transport inhibitor cocktail consisting of brefeldin A and monensin (Invitrogen, CA, USA) was added into the culture suspension to block cytokine secretion. Flow cytometry was used to examine the expression of FoxP3⁺, CD5⁺FoxP3⁺ and CD24⁺CD27⁺ as well as expression of intracellular IL-10. Viability counts were completed before and after 72 hours of culture. Before culture, the viability of B cells was

99.9±0.1%. After 72 hours of culture, the PMA-alone B cell viability was 69.1±2.3%, the media-alone B cell viability was 86.5±1.6%, and the 25pg/mL of IL-4 condition B cell viability was 82.9±1.3%.

Cell Staining and Gating Strategy for B Cells

Briefly, cells were stained with fluorescent antibodies compatible for flow cytometry analysis of B cells and B_{regs} subsets. Cells were washed twice with FACS buffer. Antibodies to surface markers were incubated with the cells for 30 minutes, and then cells were washed, fixed and permeabilized before antibodies to intracellular markers were incubated with the cells for 30 minutes. Appropriate isotypes were used as negative controls. The cells were acquired using a Becton Dickinson (BD) LSRFortessa 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 (V450), CD19 (PE-Cy7) and intracellularly with FoxP3 (Alexa Fluor 488). 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 then used to plot CD19 with FoxP3 to isolate CD19+FoxP3+ B cells in the airways.

Peripheral blood and bone marrow mononuclear cells were surface-stained with antibodies to CD19 (PE-Cy7), CD1d (APC), CD5 (Alexa Fluor 700), CD24 (PE-CF594), CD27 (V450), CD38 (PE) and intracellularly with IgE (APC), IL-10 (PE) and FoxP3 (Alexa Fluor 488). The following gating strategy was used to enumerate B_{regs} from peripheral blood and bone marrow. From the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around all the cells, excluding debris. SSC and CD19 were then plotted and a gate was placed around the CD19+ population. From the CD19+ B cell population, further gates were created to enumerate (1) CD1d+CD5+, (2) CD5+FoxP3+, (3) CD24+CD27+, and (4) CD24+CD38+ B_{reg} subsets (Figure 1).

Appropriate isotype controls were used to accurately determine expression of intracellular expression of FoxP3 and IL-10. Obtaining sufficient cell yield, especially from sputum is challenging, and minimizing the number of tubes for the experiment was necessary to enable detection of these rare cell subtypes across all compartments. Specifically, the isotype gate for each sample was set to 1% to ensure consistency of gating across all samples.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., CA, USA). Data are presented as the mean $\pm$ SEM with the exception of methacholine PC₂₀ presented as geometric mean (95% confidence interval). Cross-sectional cohort data and different culture conditions were analyzed with 1-way ANOVAs with Tukey post-hoc tests. Inhalation challenge data were analyzed with 2-way ANOVAs with Bonferroni post-hoc tests. Significance was accepted at $p < 0.05$.

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SUPPLEMENTARY TABLE AND FIGURE CAPTIONS

Supplementary Table EI: Subject demographics. All data are shown as mean±SEM except for methacholine PC₂₀ (mg/mL) shown as geometric mean (95% CI). FEV₁ - forced expiratory volume in 1 second, PC₂₀ - provocative concentration of methacholine inducing at least a 20% fall in FEV₁, * p<0.05 versus controls, # p<0.05 versus allergic subjects.

Supplementary Table EII: Allergic asthmatics response to inhalation challenge. All data are shown as mean±SEM except for MCh PC₂₀ shown as geometric mean (95% CI). FEV₁ - forced expiratory volume in 1 second, MCh PC₂₀ - provocative concentration of methacholine inducing at least a 20% fall in FEV₁, EAR – maximum % change in FEV₁ from 0-2h post-challenge, LAR – maximum % change in FEV₁ from 3-7h post-challenge, HDM – house dust mite, ND - no data, * p<0.05 versus diluent.

Supplementary Table EIII: Subject demographics. All data are shown as mean±SEM except for methacholine PC₂₀ (mg/mL) shown as geometric mean (95% CI). FEV₁ - forced expiratory volume in 1 second, PC₂₀ - provocative concentration of methacholine inducing at least a 20% fall in FEV₁.

Supplementary Table EIV: B cell and regulatory B cell frequencies of allergic asthmatic subjects after allergen inhalation challenge. All data are expressed as % of CD19⁺ B cells and shown as mean±SEM, except CD19⁺ B cells, which are expressed as a % of lymphocytes. ND - no data, * p<0.05 versus diluent

Supplementary Table EV: Frequency of regulatory B cells in blood and bone marrow after allergen challenge and the effects of inhaled allergen quantified with flow cytometry assessments of FoxP3⁺ regulatory B cell frequencies of allergic asthmatic subjects. All data are expressed as % of CD19⁺ B cells and shown as mean±SEM. ND - no data, * p<0.05 versus diluent.

Supplementary Figure E1: Representative gating strategy for regulatory B cell phenotypes with isotype controls.

154 **Supplementary Figure E2:** The frequency of CD19⁺ B cells expressed as a percentage lymphocytes
155 in (A) bone marrow (n=10), (B) blood (n=10), and (C) sputum (n=8) of allergic asthmatic subjects at
156 baseline, 7 hours and 24 hours after inhalation challenge. The frequency of FoxP3⁺ B_{regs} phenotypes
157 expressed as a percentage of CD19⁺ B cells in (D) bone marrow (n=10) of allergic asthmatic subjects
158 at baseline and 24 hours after inhalation challenge and (E) blood (n=10) at baseline, 7 hours and 24
159 hours after allergen (grey bars) and diluent (white bars).

160 **Supplementary Figure E3:** IL-4-induced intracellular expression of (A) FoxP3 and (B) IL-10 of
161 CD24⁺CD27⁺ B_{regs} in CD19⁺ B cells purified from blood of allergic asthmatic subjects, where each
162 symbol represents a single subject (n=6).

163

SUPPLEMENTARY TABLES

	Controls (n=7)	Allergic Asthmatics (n=11)	Allergics (n=7)
Gender (M/F)	3/4	6/5	1/6
Age (years)	38±5	30±4	23±3
FEV₁ (% of predicted)	95.4±1.5	94.0±3.3	96.7±3.4
Methacholine PC₂₀ (mg/mL)	>16	4.2 (2.1, 8.4)	>16
Sputum Eosinophils (%)	0.06±0.04	1.9±0.4*#	0.6±0.2
Blood Eosinophils (%)	1.3±0.3	3.9±1.1*	1.8±0.4

Supplementary Table EI: Subject demographics. All data are shown as mean±SEM except for methacholine PC₂₀ (mg/mL) shown as geometric mean (95% CI). FEV₁ - forced expiratory volume in 1 second, PC₂₀ - provocative concentration of methacholine inducing at least a 20% fall in FEV₁, * p<0.05 versus controls, # p<0.05 versus allergic subjects.

Sex	Age (years)	Predicted FEV ₁ (%)	MCh PC ₂₀ Pre-Allergen (mg/mL)	MCh PC ₂₀ Pre- Diluent (mg/mL)	MCh PC ₂₀ Post-Allergen (mg/mL)	MCh PC ₂₀ Post-Diluent (mg/mL)	Allergen Extract Inhaled	Dilution of Inhaled Extract	EAR Allergen (%)	EAR Diluent (%)	LAR Allergen (%)	LAR Diluent (%)
M	37	85.1	7.7	20.8	4.1	ND	Grass	1:512	-34.8	1.0	-12.7	4.0
F	22	79.4	1.6	5.0	1.0	2.8	Ragweed	1:8	-50.0	-0.8	-31.3	-2.0
M	24	81.0	4.0	10.0	0.88	4.8	HDM	1:128	-34.1	-5.1	-19.5	-5.1
M	23	88.9	0.3	0.6	ND	ND	Cat	1:512	-22.0	-2.3	-22.3	-7.3
F	23	94.5	7.5	5.7	0.7	ND	Horse	1:128	-47.3	-0.6	-26.5	-1.9
M	66	95.4	3.4	12.3	ND	ND	Cat	1:32	-48.5	-1.8	-38.8	0.0
M	53	97.6	5.4	10.3	7.4	7.3	HDM	1:64	-36.2	-3.8	-19.5	-1.8
M	28	112.4	10.5	9.6	0.1	ND	HDM	1:64	-49.4	-7.2	-17.7	-4.9
F	22	94.3	4.7	5.3	0.2	ND	Tree	1:32	-35.4	-2.7	-50.7	-2.7
M	51	98.4	0.3	0.4	0.8	1.7	Cat	1:64	-33.8	-4.5	-22.5	-3.1
35±5		92.7±3.1	2.8 (1.1, 7.0)	5.0 (2.0, 12.6)	0.8 (0.3, 2.8)*	4.2 (1.3, 9.9)			-39.2±2.9*	-2.8±0.8	-26.2±3.6*	-2.5±1.0

Supplementary Table EII: Allergic asthmatics response to inhalation challenge. All data are shown as mean±SEM except for MCh PC₂₀

shown as geometric mean (95% CI). FEV₁ - forced expiratory volume in 1 second, MCh PC₂₀ - provocative concentration of methacholine

inducing at least a 20% fall in FEV₁, EAR – maximum % change in FEV₁ from 0-2h post-challenge, LAR – maximum % change in FEV₁

from 3-7h post-challenge, HDM – house dust mite, ND - no data, * p<0.05 versus diluent.

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	Allergic Asthmatics (n=6)
Gender (M/F)	2/4
Age (years)	32±6
FEV₁ (% of predicted)	87.9±5.1
Methacholine PC₂₀ (mg/mL)	6.4 (0.3, 13.0)
Sputum Eosinophils (%)	1.1±0.6

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180 **Supplementary Table EIII:** Subject demographics of asthmatics donating blood for B cell cultures.

181 All data are shown as mean±SEM except for methacholine PC₂₀ (mg/mL) shown as geometric mean
 182 (95% CI). FEV₁ - forced expiratory volume in 1 second, PC₂₀ - provocative concentration of
 183 methacholine inducing at least a 20% fall in FEV₁.

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	Baseline		7 Hours Post		24 Hours Post	
	Allergen	Diluent	Allergen	Diluent	Allergen	Diluent
BONE MARROW						
CD19+	15.8±1.5	15.3±1.4	ND	ND	16.8±2.1	15.8±1.1
CD19+FoxP3+	10.5±1.6	9.3±1.6	ND	ND	5.8±1.2*	9.2±1.0
BLOOD						
CD19+	14.4±1.6	13.3±1.3	18.0±1.9	15.3±1.4	14.3±1.4	13.8±1.4
CD19+FoxP3+	7.3±1.4	10.7±1.0	15.8±4.9*	5.7±0.6	11.3±1.9	9.7±0.7
SPUTUM						
CD19+	8.6±1.3	8.0±1.6	16.7±1.6*	8.5±0.9	13.5±2.1	9.3±1.8
CD19+FoxP3+	7.4±1.2	7.8±1.1	15.5±8.0	7.9±0.9	18.7±3.0*	8.9±1.5

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186 **Supplementary Table EIV:** B cell and regulatory B cell frequencies after allergen inhalation

187 challenge. All data are expressed as % of CD19+ B cells and shown as mean±SEM, except CD19+ B

188 cells, which are expressed as a % of lymphocytes. ND - no data, * p<0.05 versus diluent.

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	Baseline		7 Hours Post		24 Hours Post	
	Allergen	Diluent	Allergen	Diluent	Allergen	Diluent
BONE MARROW - Breg Phenotypes						
CD5+	12.1±1.2	14.0±1.5	ND	ND	11.8±1.3	13.2±0.7
CD1d+CD5+	4.6±0.3	5.6±0.9	ND	ND	5.3±0.6	6.0±0.6
CD24+CD27+	4.5±0.2	5.2±0.4	ND	ND	6.5±0.9	5.4±0.3
CD24+CD38+	38.0±2.4	34.5±3.4	ND	ND	25.4±3.1	30.0±4.0
CD5+FoxP3+	0.8±0.1	1.1±0.2	0.9±0.2	0.8±0.1	1.8±0.4*	1.0±0.1
CD1d+CD5+ FoxP3+	0.32±0.05	0.42±0.06	0.25±0.06	0.31±0.04	0.66±0.19	0.31±0.05
CD24+CD27+ FoxP3+	0.8±0.2	0.8±0.2	1.3±0.4	0.5±0.1	1.2±0.4*	0.5±0.1
CD24+CD38+ FoxP3+	0.8±0.2	0.8±0.2	1.1±0.3	0.8±0.2	2.2±0.7*	0.7±0.1
BLOOD - Breg Phenotypes						
CD5+	11.5±1.3	10.6±0.9	7.2±1.4*	12.3±1.2	12.7±1.2	11.6±0.5
CD1d+CD5+	3.2±0.2	3.6±0.3	2.1±0.4*	4.3±0.6	4.4±0.4	3.9±0.4
CD24+CD27+	5.2±0.9	5.9±0.8	7.2±1.1	5.4±0.8	8.4±1.5	5.4±0.6
CD24+CD38+	7.0±1.1	7.9±1.3	9.8±1.5	8.7±1.6	14.1±2.2*	7.3±1.0
CD5+FoxP3+	0.8±0.1	1.1±0.2	0.9±0.2	0.8±0.1	1.8±0.4*	1.0±0.1
CD1d+CD5+ FoxP3+	0.32±0.05	0.42±0.06	0.25±0.06	0.31±0.04	0.66±0.19	0.31±0.05
CD24+CD27+ FoxP3+	0.8±0.2	0.8±0.2	1.3±0.4	0.5±0.1	1.2±0.4*	0.5±0.1
CD24+CD38+ FoxP3+	0.8±0.2	0.8±0.2	1.1±0.3	0.8±0.2	2.2±0.7*	0.7±0.1

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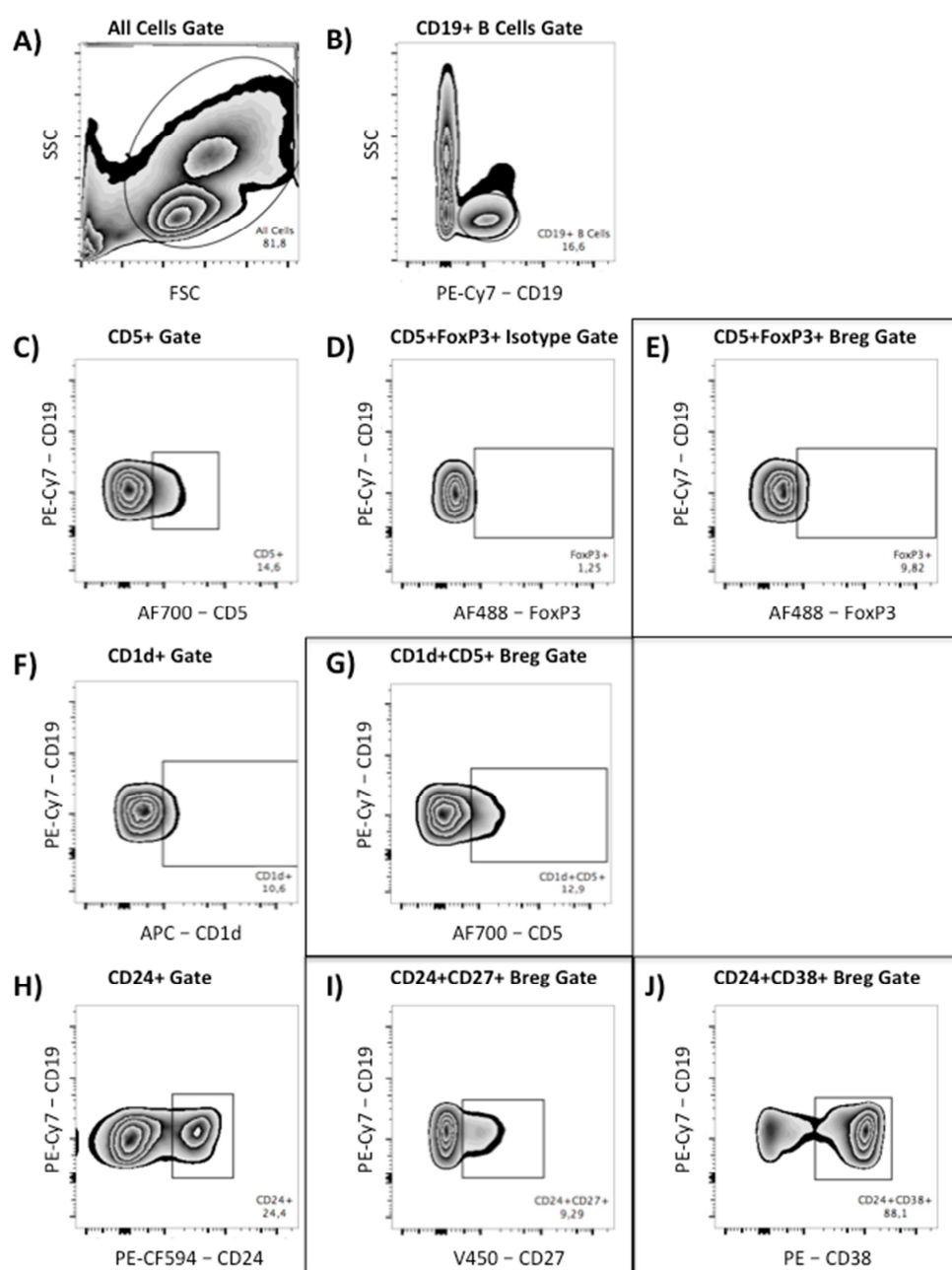
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Supplementary Table EV: Frequency of regulatory B cells in blood and bone marrow after allergen

challenge and the effects of inhaled allergen quantified with flow cytometry assessments of FoxP3+

regulatory B cell frequencies. All data are expressed as % of CD19+ B cells and shown as mean±SEM.

ND - no data, * p<0.05 versus diluent.



■ Allergen Challenge
□ Diluent Challenge

