

Original article

Allergen provocation increases TH2-cytokines and FOXP3 expression in the asthmatic lung

Background: Allergic asthma is caused by allergen-specific IgE and T-helper cell (Th) type 2 responses towards airborne allergens. The objective of this study was to investigate local and systemic regulatory mechanisms in the early asthmatic response to bronchial allergen provocation.

Methods: Birch pollen-allergic patients with mild asthma ($n = 13$) and healthy nonallergic controls ($n = 14$) were subjected to bronchoalveolar lavage (BAL) and blood sampling. On patients BAL was performed twice: without preceding provocation ('before samples') and 24 h after bronchial provocation with birch pollen allergen. Lymphocytes in BAL and peripheral blood mononuclear cells (PBMCs) were phenotyped by multi-colour flow cytometry and cytokines measured by cytometric bead array. Proliferation and secreted cytokines were analysed in allergen-stimulated PBMCs, CD25⁺ depleted PBMCs and PBMCs with IL-10 neutralizing antibodies.

Results: The numbers of CD69⁺ and FOXP3⁺ lymphocytes were higher in BAL after compared with before allergen provocation in asthmatic patients. Moreover, allergen provocation increased expression of FOXP3 in CD4⁺CD25^{bright} cells. The cytokine profile in BAL fluid from asthmatics revealed higher levels of IL-5, compared with the controls, and an increase in IL-5, IL-6, IL-9 and IL-10 after allergen provocation. Pollen allergen stimulated PBMC cultures from asthmatic patients produced elevated levels of IL-5 and IL-13 compared with the controls, which were not affected by depletion of CD25⁺ cells or IL-10 neutralization.

Conclusion: Despite an increase in CD4⁺CD25^{bright} cells expressing high levels of FOXP3 in response to bronchial allergen provocation, asthmatic patients exhibit enhanced levels of Th2 cytokines in the lung, which may indicate an inability among infiltrating cells to suppress Th2 responses.

**S. Thunberg^{1*}, G. Gafvelin^{1*},
M. Nord², R. Grönneberg²,
J. Grunewald², A. Eklund²,
M. van Hage¹**

¹Clinical Immunology and Allergy Unit, Department of Medicine, Karolinska Institute; ²Division of Respiratory Medicine, Department of Medicine, Karolinska Institute and University Hospital, Stockholm, Sweden

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Guro Gafvelin
Department of Medicine
Clinical Immunology and Allergy Unit
Karolinska Institute
Karolinska University Hospital
L2:04
SE-17176 Stockholm
Sweden

*Shared first authorship

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Allergic asthma is an inflammatory disease caused by inappropriate responses to inhaled allergens. It is characterized by allergen-specific IgE and secretion of the Th2 cytokines IL-4, -5, -9 and 13 by T-helper cells (Th) with subsequent activation of mast cells, infiltration of eosinophils and airway smooth muscle constriction (1). Furthermore, over-expression of GATA-3, the lineage differentiation marker for Th2 cells, results in enhanced Th2-effector cell function in mice and humans and asthma-like symptoms in mice (2).

CD4⁺CD25^{bright} regulatory T-cells (Treg), expressing FOXP3, regulate proliferation and effector functions of a number of different cell types (3). Th1 cells are more susceptible to suppression by Treg cells than Th2 cells (4) and it was recently shown that GATA3 inhibits FOXP3-expression through binding to the FOXP3 promoter, thus preventing Th2-cells at various stages of differentiation from expressing FOXP3 (5). This might explain the defective suppression of allergen-specific responses by

Treg cells isolated from peripheral blood that we (6) and others (7, 8) have previously reported. Most studies on immune regulation are performed on cells obtained from peripheral blood, which may not reflect the situation in the target organ. Thus, studies on the target organ of allergic asthma, i.e. the lungs, are essential to perform. Hartl et al. recently reported that decreased numbers of pulmonary CD4⁺CD25^{bright} Treg cells were obtained from asthmatic compared with nonasthmatic children and that isolated pulmonary CD4⁺CD25^{bright} cells from the asthmatic children failed to suppress pulmonary Th2 responses (9).

Here we sought to study the allergen specific T-cell response and Treg function in blood *in vitro*, the phenotype of pulmonary and blood Treg and T-effector cells *ex vivo* and the cytokine profile in bronchoalveolar lavage (BAL) fluid during allergen induced asthmatic responses. The aim was to elucidate the role of T-effector and Treg cells in the early allergen induced asthmatic reaction.

Methods

Study population and design

Thirteen subjects with mild allergic asthma to birch pollen (age 22–42 years, seven females) and 14 nonallergic control subjects (age 23–46 years, eight females) participated. All asthmatic patients had allergen-specific IgE to birch pollen (≥ 2 kU/l), while nonallergic controls were negative (≤ 0.35 kU/l) to Phadiatop (a mix of common inhalant allergens, Immuno CAP System, Phadia AB, Uppsala, Sweden). On entering the study, subjects were

asymptomatic, their asthma had been stable with only occasional medication with inhaled β_2 -agonists. The wash-out period for inhaled steroids was at least four weeks and for anti-histamines, anti-leukotrienes and NSAIDs minimum two weeks. No exacerbations or airway infections were allowed during the last four weeks before sampling. Clinical data, patient characteristics and sample collection are presented in Table 1. A detailed description on the clinical parameters will be published elsewhere.

Bronchoscopy and bronchoprovocation was performed outside the pollen season. At least 2 weeks after the first bronchoscopy

Table 1. (A) Clinical characteristics of asthmatics and controls – overview. (B) Asthmatic patients – individual characteristics and sample collection. (C) Controls – individual characteristics and sample collection

(A)										
						Asthmatics		Controls		
Subjects, <i>n</i>						13		14		
Sex (F/M)						7/6		8/6		
Age (mean, [range])						30 [22–42]		29 [23–46]		
FEV1% (mean ± SD)						103 ± 12		107 ± 8.5		
Metacholine challenge PD20 (mean ± SD)						437 ± 551		all negative		
Phadiatop (% positive)*						100		0		
Allergen challenge SQ PD20 (mean ± SD)						870 ± 908		ND		
Allergen challenge, maximal drop in FEV1% (mean ± SD)						30.7 + 12.2		ND		
(B)										
Patient	Sex	Age†	IgE‡ birch	IgE‡ timothy	IgE‡ others	BAL fluid cytokines§	BAL cells§	Blood cells§	PBMC culture§	RNA analysis§
1	F	42	8	0	0	X	X	X		
2	F	38	47	12	cat 11		X	X		
3	F	36	31	5	ragweed 3	X	X	X	X	X
4	M	22	20	0	dog 8	X	X	X	X	X
5	F	29	51	0	ragweed 1	X	X	X	X	X
6	F	32	4	7	horse 12	X	X	X	X	X
7	F	34	3	0	cat 8	X	X	X	X	X
8	M	25	54	20	cat 2	X	X	X	X	X
9	M	28	48	5	cat 3, dog 2			X	X	X
10	F	36	28	17	cat 13	X	X	X	X	X
11	M	24	2	1	mites 1	X			X	
12	M	24	16	2	ragweed 1	X	X	X	X	X
13	M	24	>100	>100	horse >100, cat 20	X	X	X	X	X
(C)										
Control	Sex	Age†	IgE‡ birch	IgE‡ timothy	IgE‡ others	BAL fluid cytokines§	BAL cells§	Blood cells§	PBMC culture§	RNA analysis§
1	M	38	0	0	0	X				
2	F	36	0	0	0	X				
3	F	46	0	0	0	X	X	X		
4	M	37	0	0	0	X	X	X		
5	M	25	0	0	0	X	X	X	X	X
6	F	25	0	0	0	X	X	X	X	X
7	F	26	0	0	0	X	X	X	X	X
8	F	26	0	0	0	X	X	X	X	X
9	F	23	0	0	0	X		X	X	X
10	M	25	0	0	0	X	X	X	X	X
11	F	23	0	0	0	X	X	X	X	X
12	F	24	0	0	0	X	X	X	X	X
13	M	26	0	0	0	X	X	X	X	X
14	M	25	0	0	0	X		X		X

F, female; M, male; FEV1%, forced expiratory volume in one second – percent of predicted normal; PD20, provocative dose causing a 20% fall in FEV1; SD, standard deviation; ND, not done; SQ, arbitrary units relating the concentration of allergen.

*IgE to at least one inhalant allergen, †years, ‡kU/l, §X indicates that samples have been analysed from patients (both before and after allergen provocation) or controls (on a single occasion, without preceding provocation).

(range 14–98 days), the asthmatic individuals were subjected to bronchoprovocation with increasing dosages of birch pollen extract (Aquagen SQ, ALK, Copenhagen, Denmark), using a dosimeter-controlled jet nebulizer (Spira Electro 2®; Respiratory Care Center, Hameenlinna, Finland) according to previously described protocols (10) to reach a final drop in FEV₁ ≥ 20% from the postdiluent baseline value. Eighty ml of heparinized peripheral blood was collected in connection to all bronchoscopies. All participants gave their informed consent. The study was approved by the local ethics committee.

Bronchoalveolar lavage samples

Bronchoalveolar lavage (BAL) was carried out as described previously (11). BAL cells and fluid were harvested from controls (control samples) and patients after the first ('before' samples, i.e. without prior allergen provocation) and second BAL ('after' samples, i.e. 24 h after allergen provocation). The BAL fluid was strained through a Dacron net (Millipore, Bedford, Ireland) and centrifuged at 400 *g* 10 min at 4°C. BAL cells were counted and analysed for viability by Trypan Blue exclusion (93% (84–98%) [mean (range)] for before samples, 90% (82–98%) for after samples, 94% (86–100%) for controls). BAL fluid was kept at –80°C until use. Before cytokine analysis, the BAL fluid samples were concentrated 60–830x on Amicon Ultra filters (Millipore) with 3K cutoff.

PBMC cultures

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation. CD25⁺ cells were depleted from 10 × 10⁶ PBMCs using anti-CD25 labelled magnetic beads, according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The CD25-depleted fraction typically contained 2.8% (1.1–4.1) CD4⁺FOXP3⁺ cells, compared to 67.2% (58.6–77.9) in the removed CD25⁺ fraction, as analysed in three control experiments.

Pollen allergen ('pollen-mix' containing 1 µg/ml of birch pollen extract and 0.2 µg/ml of grass pollen extract (Aquagen SQ)) stimulated PBMCs, CD25-depleted PBMCs and PBMCs with an added neutralizing anti-IL-10 antibody (5 µg/ml, Mabtech, Nacka, Sweden) was cultured for six days for proliferation and cytokine analysis, as described elsewhere (12).

Cytometric bead array (CBA)

IL-5, IL-6, IL-9 (only in BAL fluid), IL-10, IL-13 (only in PBMC supernatants), IFNγ and TNFα were measured in PBMC culture supernatants and BAL fluid using cytometric bead array (CBA) Flex Set (BD Biosciences, San Jose, CA, USA). Samples were run on a FACSaria flow cytometer and data analysed using FCAP Array software (BD Biosciences). Detection range 2–5000 pg/ml. Analyses of samples containing > 5000 pg/ml were repeated after dilution. Cytokine concentrations in nonconcentrated BAL fluid were calculated using the concentration factors of BAL fluid samples. As the concentration factors varied between individual samples, the detection limits had to be adjusted to correct for false negative results in less concentrated BAL samples. Thus, the detection limits were set to the calculated concentration from the least concentrated BAL sample with detectable level of each cytokine: for IL-5, 10 fg/ml; IL-9, 25 fg/ml; IFNγ, 2 fg/ml; TNFα, 15 fg/ml (IL-6 and IL-10 were detected in all samples). Calculated concentrations below these values were set to the detection limit.

Flow cytometry

Freshly prepared PBMCs or BAL cells resuspended in PBS or PBS with 0.5% AB-serum, respectively, were subjected to six-colour FACS analysis. A total of 1 × 10⁶ cells/panel were stained with the following antibodies: FITC-conjugated anti-human CD69, 6B11 invariant NKT, CD16 or CD11a (BD Pharmingen, San Jose, CA, USA), PE-conjugated Vβ11 (Immunotech, Marseille, France), CD56 or OX40 (CD134), PECy5-conjugated CD45RO, CD161, CXCR3 or HLA DR, PECy7-conjugated CD14, APC-conjugated CD25, CD8 or CD40L (CD154), APCy7-conjugated CD4 and Pacific blue-conjugated CD3 (BD Pharmingen). IgG1-FITC, IgG2a-PE, IgG2a-PECy5, IgG2a-PECy7 and IgG1-APC were used as isotype controls (BD Pharmingen). FOXP3 was stained with PE-conjugated anti-FOXP3 (clone PCH101) using a FOXP3 intracellular staining kit (eBioscience, San Diego, CA, USA). Stained cells were analysed on a FACSaria flow cytometer (BD Biosciences) with FACS-DIVA software.

Real-time PCR

RNA was isolated from 3 × 10⁶ un-stimulated or anti-CD3/CD28 stimulated PBMCs (48 h) in AIM-V medium (Gibco, Paisley, UK) with RNeasy Mini kit (Qiagen, Hilden, Germany) followed by reverse transcription to cDNA (Fermentas, St. Leon-Rot, Germany). Amplification of cDNA was made in triplicates with SYBR green supermix using iCycler IQ sequence detection system (Bio-Rad) with primers for T-bet, GATA3, FOXP3 (13), RORC2 (14) and house keeping gene EF1A (13) (synthesized by CyberGene AB, Stockholm, Sweden). The Ct of target genes were normalized to EF1A (ΔCt) for stimulated and un-stimulated cells. Relative quantification and calculation was performed using the comparative threshold cycle method (2^{–ΔΔCt}).

Statistics

The Mann–Whitney *U*-test was used for comparisons between controls and asthmatics and Wilcoxon matched pairs test for comparisons within groups. GraphPad Prism version 4.03 (Graphpad Software Inc, San Diego, CA, USA) was used for the calculations.

Results

Lymphocyte antigen expression on BAL cells and PBMCs

Antigen expression on gated lymphocytes was analysed for BAL cells and PBMCs obtained from asthmatics before and after allergen provocation or from controls (results summarized in Table 2). As expected CD3⁺ T-cells were more frequent in blood than in BAL, while the percentage of activated CD3⁺CD69⁺ T-cells was higher in BAL than in PBMCs. Moreover, the percentage of cells positive for the NKT cell markers Vβ11 and 6B11 was higher in BAL than in blood for controls as well as asthmatics before and after allergen provocation (*P* = 0.02, 0.008 and 0.04, respectively). There was no difference in the CD4⁺/CD8⁺ ratio between BAL and PBMCs (not shown). More Th1-associated CD4⁺CXCR3⁺ T-cells (15) were detected in BAL than in PBMCs (*P* = 0.03, 0.004 and 0.004 for

Table 2. Lymphocyte antigen expression in BAL cells and peripheral blood

	Control BAL (%)	Before BAL (%)	After BAL (%)	Control PBMC (%)	Before PBMC (%)	After PBMC (%)
CD3 ⁺ of total cells	4.5 (2.1–11.5)	5.2 (1.7–11.6)	3.7 (1.2–11.5)	22.5 (9.8–38.9)	31.4 (9.1–51.9)	25.7 (3.2–54.7)
NKT	0.4 (0.1–0.9)	0.3 (0.1–1.5)	0.5 (0–1.6)	0.065 (0.02–0.3)	0.02 (0–0.66)	0.02 (0–0.5)
CD3 ⁺ CD69 ⁺	37.7 (23.9–70.6)	42.5 (20.9–63.8)	54.4*† (32.5–63.6)	0.8 (0.2–2.9)	0.6 (0.1–1.8)	0.8 (0.2–2.5)
CD4 ⁺ CXCR3 ⁺	59.2 (6.7–76.8)	52.3 (30–80.3)	55.3 (12.6–70.5)	14.6 (4.6–38.5)	17.9 (4.5–33.4)	13.2 (3.1–30.5)
CD4 ⁺ CCR4 ⁺	0.6 (0–4.5)	0.4 (0.1–3.7)	0.8 (0–10)	0.3 (0–2.7)	0.3 (0.1–1.4)	0.1*†‡ (0–0.4)
CD4 ⁺ CD25 ⁺	10 (2.8–13.4)	8.4 (1.6–29.8)	9.3 (4.4–27.6)	9.5 (2.6–15.8)	6.9 (1.0–28.1)	10.5*† (1.4–48.9)
CD4 ⁺ FOXP3 ⁺	7.6 (2.8–15.8)	7.8 (3.2–25.9)	13.9**†‡ (7.9–28.4)	5.4 (1.6–15.1)	5.5 (1.9–9.1)	5.5 (3–11.8)

All cell types except for CD3⁺ refer to cells in the lymphocyte gate. NKT cells are CD3⁺Vb11⁺6B11⁺. Data are from ten controls and eleven asthmatics (before and after provocation) for BAL cells, and twelve controls and thirteen asthmatics (before and after) for PBMCs, as detailed in Table 1.

* $P < 0.05$, ** $P < 0.01$.

Values are given as medians and ranges. Comparisons between: †asthmatics before and after provocation (Wilcoxon matched pairs test); ‡controls and asthmatics after provocation (Mann–Whitney U -test).

controls, asthmatics before and after provocation, respectively) and the Th2-associated chemokine receptor CCR4 (15) was expressed on a larger proportion of CD4⁺ T-cells in BAL from controls ($P = 0.03$) and asthmatics after bronchial allergen provocation ($P = 0.008$). The Treg-associated transcription factor FOXP3 was expressed in a larger proportion of BAL cells compared with peripheral blood from asthmatics ($P = 0.008$ and 0.004 , respectively before and after provocation).

Staining with the lymphocyte early activation marker CD69 revealed an increase of CD3⁺CD69⁺ cells in BAL after allergen provocation compared with that before allergen provocation ($P = 0.04$). Moreover, CD4⁺FOXP3⁺ cells increased in BAL after allergen provocation ($P = 0.004$). Compared with the controls the percentage of CD4⁺FOXP3⁺ BAL cells was significantly elevated in asthmatics after ($P = 0.008$) but not before allergen provocation. Consequently, both activated CD3⁺CD69⁺ lymphocytes and FOXP3⁺ cells increase in BAL after provocation, while in PBMCs the numbers of CD69 and FOXP3 positive cells were not affected by provocation or different between asthmatic patients and the controls (Table 2).

Allergen provocation leads to increased expression of FOXP3 in BAL CD4⁺CD25^{bright} cells

To further analyse the CD4⁺FOXP3-expressing cells infiltrating the lung, CD4⁺ T-cells from BAL and peripheral blood were divided into two subpopulations based on their expression of CD25 (Fig. 1A), and the mean fluorescent intensity (MFI) of FOXP3-expression was analysed. The CD4⁺CD25^{bright} cells in BAL and PBMCs from both healthy controls and asthmatic patients were significantly brighter in their expression of FOXP3, compared with CD4⁺CD25^{intermediate} T-cells. Moreover, expression of FOXP3 increased in CD4⁺CD25^{bright} BAL cells obtained from asthmatic patients after bronchial allergen provocation, compared with CD4⁺CD25^{bright} cells obtained before provocation ($P = 0.03$) as well as to the healthy controls ($P = 0.005$)

(Fig. 1B). Thus, allergen provocation resulted in elevated expression of FOXP3 in pulmonary CD4⁺CD25^{bright} cells.

IL-5, IL-6, IL-9 and IL-10 increase in BAL fluid after allergen provocation

Cytokine measurements revealed higher levels of IL-5 in BAL fluid from asthmatic patients (before provocation) than from healthy controls ($P = 0.021$), Fig. 2. Allergen bronchoprovocation increased the levels of IL-5 ($P = 0.0005$), IL-6 ($P = 0.034$), IL-9 ($P = 0.031$) and IL-10 ($P = 0.014$) in asthmatic patients, resulting in

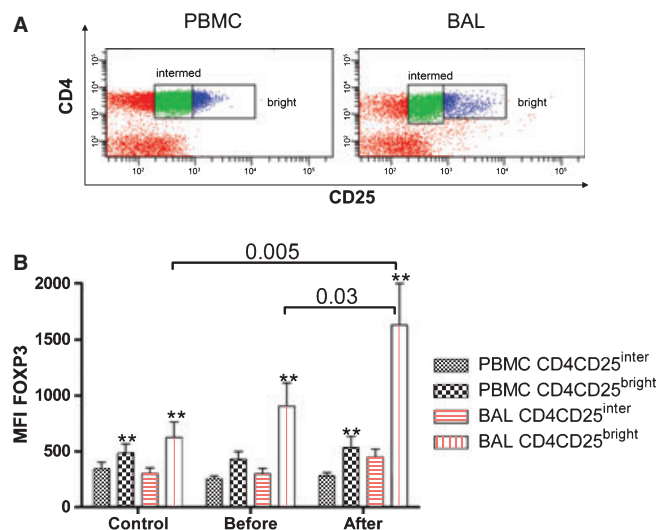


Figure 1. FOXP3 expression increase in CD4⁺CD25^{bright} cells after allergen provocation. (A) CD4⁺CD25⁺ cells in blood and BAL were divided into CD4⁺CD25^{inter} (intermediate) and CD4⁺CD25^{bright} cells. (B) Mean fluorescence intensity of intracellular FOXP3 in PBMCs and BAL cells from 10 controls and 11 asthmatic patients, before and after allergen provocation for the asthmatic patients. Mean with SEM, **($P < 0.01$) refers to comparisons between CD4⁺CD25^{inter} and CD4⁺CD25^{bright} cells. Exact P -values are for indicated comparisons between groups.

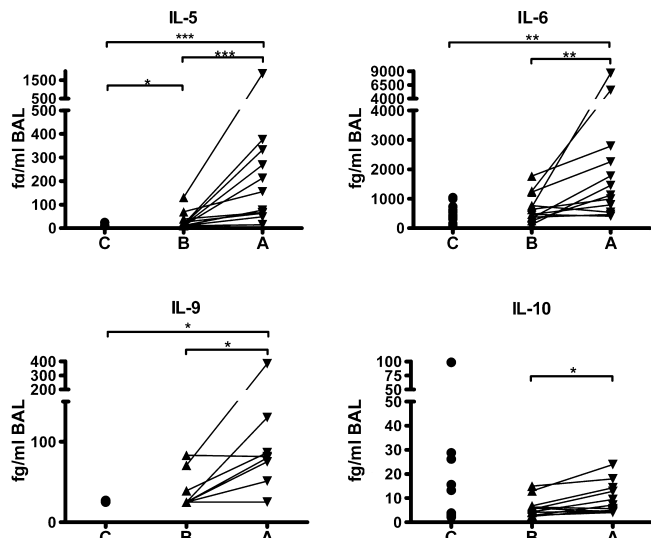


Figure 2. Enhanced levels of IL-5, IL-6, IL-9 and IL-10 in BAL fluid of asthmatic patients after allergen provocation compared with that before allergen provocation. Cytokine concentrations in BAL fluid (fg/ml) after adjustment for concentration of analysed samples as described in Methods. BAL fluid from 12 controls (C), and 12 asthmatics before (B) and after (A) allergen provocation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

significantly higher concentrations of IL-5 ($P < 0.0001$), IL-6 ($P = 0.022$) and IL-9 ($P = 0.028$), but not IL-10, compared with the healthy controls. By contrast, TNF α and IFN γ levels in BAL did not differ between asthmatic patients and controls and were not affected by allergen provocation (not shown). In most samples, the IFN γ values were below the detection level.

The data show that allergen provocation leads to increased levels of IL-5, IL-6, IL-9 and IL-10 in the asthmatic lung, but has no effect on the proinflammatory/Th1 cytokines TNF α and IFN γ .

Increased FOXP3 mRNA expression after allergen provocation

To map Th1, Th2, Treg as well as Th17 cells in response to allergen provocation, mRNA expression of the lineage

markers T-bet, GATA3, FOXP3 and RORC2 was quantified in antiCD3/antiCD28 stimulated PBMCs. When comparing healthy controls and allergic asthmatics before allergen provocation, no significant difference in mRNA expression of any of the examined genes was detected. The expression relative to house keeping gene EF1A of T-bet, GATA3 and RORC2 was in general low with median values between 0.63 and 1.16 (Fig. 3). For the asthmatics, the median mRNA expression increased for all lineage markers after allergen provocation, compared with that before allergen provocation. Median relative change values increased from 10.89 to 15.63 for FOXP3, from 1.06 to 1.54 for T-bet, from 0.63 to 1.13 for GATA3 and from 0.78 to 1.05 for RORC2. Only the increase for FOXP3 was significant ($P = 0.048$, Fig. 3). Bronchial allergen provocation thus increased the mRNA expression of FOXP3.

Depletion of CD4⁺CD25⁺ T-cells affects production of Th1 cytokines in allergen stimulated PBMC cultures

We next examined whether proliferation and cytokine production by allergen-specific effector cells was suppressed by CD3⁺CD25⁺ cells or the regulatory cytokine IL-10.

Depletion of CD4⁺CD25⁺ T-cells or neutralization of IL-10 did not affect pollen-stimulated proliferation (Fig. 4A). In addition, the proliferation did not differ between allergic asthmatics and the healthy controls. Instead, as shown in Fig. 4B, we analysed cytokines in the cell culture supernatants. Upon pollen allergen stimulation, PBMCs obtained from asthmatic patients before allergen provocation produced significantly more IL-5 and IL-13, compared with the healthy controls ($P = 0.004$ and 0.011 , respectively). Higher levels of IL-10 was produced by CD4⁺CD25⁺ depleted PBMCs from controls than from asthmatics ($P = 0.048$ compared with asthmatics both before and after provocation).

In the control group, depletion of CD4⁺CD25⁺ T-cells in pollen allergen stimulated PBMC cultures increased the production of TNF α and IFN γ ($P = 0.014$ and 0.047 respectively), but not IL-5, 6, 10 or 13 (Fig. 4B). Depletion of CD4⁺CD25⁺ T-cells increased production of IFN γ for

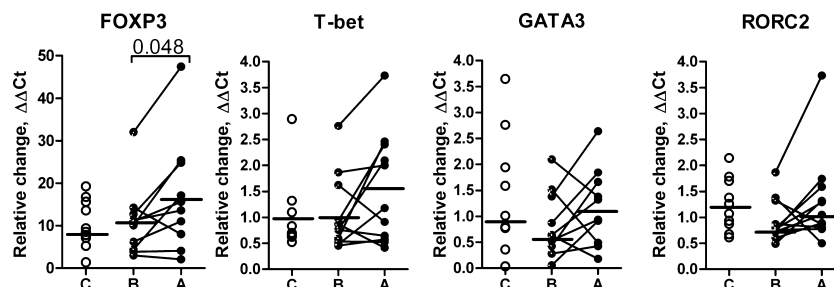


Figure 3. Increased FOXP3 mRNA expression in PBMCs from asthmatic patients after allergen provocation. FOXP3, Tbet, GATA-3 and RORC mRNA expression in antiCD3/CD28 stimulated PBMCs from controls (C, $n = 10$), asthmatics ($n = 10$) before (B) and after (A) allergen provocation expressed as relative change ($\Delta\Delta Ct$) in Ct of target genes for stimulated and un-stimulated cells after normalization to the house keeping gene EF1A. P -value indicated.

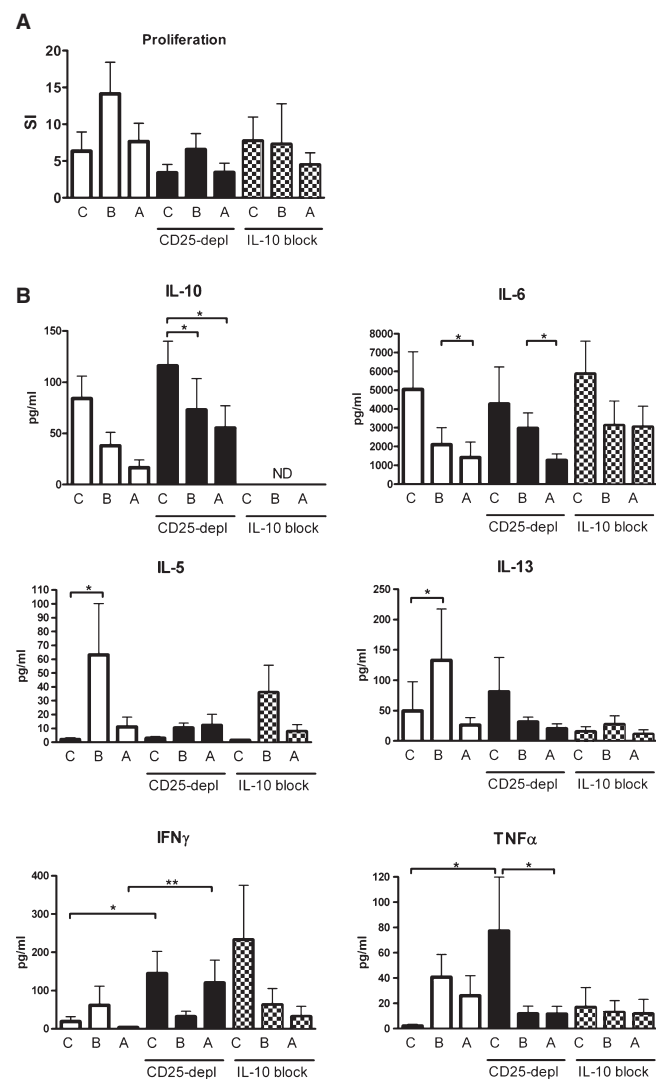


Figure 4. Th2 cytokines IL-5 and IL-13 are not affected by depletion of CD4⁺CD25⁺ cells or neutralization of IL-10 in PBMC cultures. Allergen *in vitro* stimulated PBMCs, PBMCs depleted of CD4⁺CD25⁺ cells or PBMCs with a blocking IL-10 antibody from controls (C, *n* = 9), asthmatics (*n* = 11) before (B) and after (A) allergen provocation. (A) Proliferation expressed as stimulation index (SI). (B) Cytokine concentrations (pg/ml) in supernatants from pollen stimulated cultures subtracted by the concentration in unstimulated cultures. Mean with SEM, **P* < 0.05, ***P* < 0.01.

asthmatic patients after allergen provocation (*P* = 0.008). Moreover, blocking of IL-10 in pollen stimulated PBMC cultures from controls resulted in elevated IFN γ levels (not significant, *P* = 0.055). However, IL-10 blocking had no significant effect on either Th1 or Th2 cytokines in PBMC cultures from asthmatic patients before or after allergen provocation. Hence, depletion of CD4⁺CD25⁺ T-cells in pollen-stimulated PBMC cultures increased production of Th1-, but not Th2-cytokines, indicating that CD25⁺ cells may be less effective in their suppression of Th2- than of Th1-cytokines.

Discussion

In this study, we examined the effect of allergen provocation on allergen-specific responses in blood and on cell and cytokine responses in the target organ of patients with mild allergic asthma. As control, blood and BAL was also obtained from healthy nonallergic individuals. The study design focuses on early events in the asthmatic reaction rather than the chronic inflammatory state.

Low numbers of V β 11⁺6B11⁺ NKT cells (<1% of lymphocytes) were detected in BAL from both asthmatic patients and healthy controls, in contrast to data reported by Akbari et al., showing dramatically higher numbers of pulmonary NKT cells (approximately, 60% of CD4⁺CD3⁺ cells) in patients with moderate to severe asthma (16). Thus, our results do not support a significant role for NKT cells in patients with mild asthma, consistent with data from Vijayanand et al. (17). Upon allergen provocation CD4⁺FOXP3⁺ cells and CD3⁺CD69⁺ cells increased in BAL indicating that both FOXP3⁺ putative Treg cells and recently activated CD69⁺ T-cells infiltrate the asthmatic lung upon allergen exposure. FOXP3 as a marker for human Treg cells has some limitations as T-effector cells transiently express FOXP3 after activation (18–22). Additional means to discriminate Treg cells from activated T-effector cells have been proposed e.g. CD127 negativity, which was introduced after the initiation of our study (23, 24). FOXP3 expression has been shown to correlate to suppressive function with lower expression in T-effector cells and higher levels in CD25^{bright} Treg cells and therefore we utilized the level of FOXP3 expression to more reliably identify Treg cells. CD4⁺CD25^{bright} cells intracellularly stained for FOXP3 displayed significantly higher fluorescence intensity after, compared with before allergen provocation. This finding indicates that the observed increase in infiltrating FOXP3⁺ cells corresponds to an increase of functional Treg cells. Our data differ from the results obtained by Hartl et al. showing lower levels of CD4⁺CD25^{bright} cells and FOXP3 mRNA expression in BAL cells from asthmatic children than from healthy controls or children treated with inhaled corticosteroids (9). The patients examined in that study were children with recurrent moderate to severe asthma, with elevated total and allergen-specific IgE in a majority of the children, while the patients in our study were adults with mild asthma caused by pollen allergy. Therefore, the discrepancy probably reflects differences between a chronic asthmatic state compared with an early allergic asthmatic reaction. Our data suggest that Treg cells are recruited to the lung as part of an early response to allergen provocation to control the inflammatory asthmatic response.

To further explore the suppressive function of Treg cells, we analysed *in vitro* stimulated PBMCs obtained at each experimental condition. Allergen provocation led to an increase of FOXP3 mRNA expression suggesting induction of regulatory responses during active asthmatic reactions. This is supported by data from Lee et al.

showing that FOXP3 RNA expression was higher in paediatric patients with bronchial asthma than patients with allergic rhinitis and the expression correlated with severity of the disease (25). In agreement with several other studies, allergen stimulation led to Th2 cytokine production, IL-5 and IL-13, in PBMCs from the birch pollen allergic patients (6, 26–28). These cytokines were not suppressed by CD4⁺CD25⁺ Treg cells as shown in experiments on allergen stimulated PBMC cultures depleted of CD25⁺ cells. Our data are in line with earlier studies showing that CD4⁺CD25⁺ Treg cells are not as effective in their suppression of Th2 as of Th1 responses (4, 6, 29), providing an explanation for the inability of infiltrating Treg cells to suppress Th2 driven asthmatic responses to allergen provocation in the lung. In addition it has been shown that CD4⁺CD25^{bright} cells isolated from PBMCs of allergic asthmatic children display impaired suppressive function compared with healthy children or allergen-specific immunotherapy treated asthmatic children (30).

The cytokine milieu in allergen-challenged lungs of asthmatic patients, as well as in un-exposed lungs of patients and controls, was studied by analysing cytokine levels in BAL fluid. Higher levels of IL-5 were found in BAL from asthmatic patients than from controls even in the un-exposed lung, confirming earlier studies showing that allergic asthmatic patients exhibit a Th2 skewed milieu in the lung (31, 32). Moreover, allergen provocation led to enhanced levels of IL-5, IL-6, IL-9 and IL-10. The pro-inflammatory cytokine TNF α was not elevated after allergen provocation, which could be expected considering that the examined patients all had mild asthma and that sampling was carried out after a single allergen provocation outside the pollen season. The observation that the levels of IL-9 and IL-10 were enhanced in response to allergen provocation is of particular interest in the light of a newly identified CD4⁺ T-effector cell subset expressing these cytokines but not GATA-3 (33, 34). These pro-inflammatory cells were identified in mice and could be derived from Th2

cells when stimulated in the presence of TGF β and IL-4. Previous studies have shown that IL-9 is produced mainly by CD3⁺ cells in bronchial tissue of asthmatic individuals (35) and by BAL lymphocytes after bronchial allergen provocation (36). Thus, IL-9 in BAL fluid probably originates from T-cells and these cells may correspond to the IL-9⁺IL-10⁺ Th subset identified in mice. Our data then identifies a role for these cells in the early events of the asthmatic reaction in the human lung.

We conclude that activated lymphocytes infiltrate into the lung upon allergen provocation, promoting a pro-inflammatory Th2 cytokine profile in the target organ. Simultaneously putative Treg cells expressing high levels of FOXP3 increase as part of the early inflammatory reaction to allergen exposure. We show that Th2 cytokine responses to allergen *in vitro* stimulation of PBMCs are not suppressed by CD4⁺CD25⁺ cells, suggesting that the infiltrating FOXP3⁺ cells are not able to control the allergen-induced Th2 response in the asthmatic lung.

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