

Increased aeroallergen-specific interleukin-4-producing T cells in asthmatic adults

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Summary

Background Asthma, atopy and some forms of respiratory syncytial virus (RSV) disease are thought to be caused by T cells making IL-4 (Th2 cells). However, not all patients with similar patterns of clinical disease have the same underlying pathogenesis and the ability to detect immunopathogenic T cells by examination of the peripheral blood remains in doubt. With the prospect of specific immunotherapy for diseases caused by T cell subsets, it is important to determine whether peripheral blood mononuclear cell (PBMC) reactivity can be used to establish the presence of immunopathogenic responses and therefore to predict therapeutic effects.

Objective To detect IL-4 and IFN- γ production as markers of Th1 and Th2 responses in the peripheral blood of atopic and asthmatic adults.

Methods PBMC from 22 adult asthmatics (18 of whom were atopic) and 21 non-asthmatic volunteers (ten of whom were atopic) were stimulated with cat, birch and house dust mite allergens, human rhinovirus, RSV and recombinant chimaeric F/G protein from RSV *in vitro*. ELISPOT assays were used to enumerate cells producing IL-4 and IFN- γ .

Results Asthmatics had a sixfold increase in frequencies of IL-4-producing cells to cat and birch allergen (median values: 37 vs. 7 per million PBMC, $P < 0.01$ and 20 vs. 3 per million PBMC, $P < 0.04$, respectively) compared to non-asthmatics. By contrast, non-asthmatic atopics showed no specific increase in antigen-specific IL-4 responses and there was no evident correlation between skin prick test reactivity and ELISPOT results. Atopics had significantly more IFN- γ -producing cells specific for FG than nonatopics, while IFN- γ and IL-4 responses to other antigens were not significantly different.

Conclusion Enhanced IL-4 responses to non-viral aeroallergens are seen in adults with asthma, while enhanced IFN- γ responses to viral antigen FG were seen in atopics. In practical terms, ELISPOT assays for specific cytokines may provide a method that could be used to monitor antigen-specific T cell responses in peripheral blood.

Keywords allergen, asthma, atopy, ELISPOT, IFN- γ , IL-4, respiratory syncytial virus, rhinovirus, SPT

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Introduction

Local and systemic eosinophilia and increased IgE production are seen in many patients with asthma and atopic disease. An increase in T cells making IL-4 (Th2 cells) and/or a reduction in those making IFN- γ (Th1 cells) are thought to underlie these responses. However, detecting specific Th1/2 shifts in the peripheral blood has proved difficult and conflicting observations have been reported, including increased expression of IL-4 mRNA in asthma [1], increased levels of IL-4 proteins in bronchoalveolar lavage (BAL) [2], high frequencies of Th2 cells in the peripheral blood of children with asthma, but not in adults [3], and increased IFN- γ in CD3⁺ T cells in BAL but not in the peripheral blood of adult asthmatics [4]. After non-specific stimulation, CD8 T cells from asthmatics make

more IFN- γ than those from non-asthmatic controls do, while enhanced IL-4 and IFN- γ production are both evident in cultured supernatants from asthmatic peripheral blood mononuclear cells (PBMC) [5]. We have recently observed increased IFN- γ in peripheral blood CD4⁺ and CD8⁺ T cells from asthmatic subjects [6].

The role of virus and allergen-specific T-cells in the immunopathogenesis of asthma is also unclear. Severe respiratory syncytial virus (RSV) bronchiolitis in the first year of life is associated with an increased incidence of recurrent wheeze and asthma diagnosis in later childhood [7] and we have recently shown increased frequency of RSV and cat allergen-specific IL-4-producing T cells in the PBMC of ex-bronchiolitic children age 7 to 8 years [8]. It has recently become possible to test the effects of monoclonal antibody to IL-5, IL-12 and anti-IgE in humans. Although anti-IL-5 and IL-12 both cause profound inhibition of eosinophilia, neither is effective in reducing bronchial reactivity to allergen or histamine [9, 10]. By contrast, anti-IgE therapy appears beneficial to asthmatics [11]. These reports show the need for a better understanding of asthma and allergic

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disease, and of new tests that could be used clinically to predict responses to specific immunotherapeutic trials. We therefore examined the use of ELISPOT technology to detect the frequency of T cells producing type 1 and type 2 cytokines in response to the most common allergens and viruses implicated in the pathogenesis of asthma and respiratory viral infections.

Materials and methods

Subjects

Patients and staff of the Chest and Allergy Clinic at St. Mary's Hospital were screened by the standard questionnaire and skin prick tests. Individuals were classified as 'asthmatic' based on the guidelines used by the WHO Workshop 'Global Strategy for Asthma Management and Prevention' (1995) and according to NHLBI/WHO guidelines [12], on the basis of positive symptoms by questionnaire and objective evidence of reversible airways obstruction or airway hyper-reactivity (diurnal variation in peak flow greater than 15%, bronchodilator reversibility of greater than 15% FEV₁, reduction of FEV₁ of greater than 15% after exercise testing, PC₂₀ histamine of less than 8 mg/mL). Patients were classed as 'atopic' on the basis of a > 3 mm response to at least one antigen in the skin prick test panel. Venous blood samples were obtained from a total of 63 volunteers. Twenty samples were rejected before statistical analysis because of contamination or errors or lack of response to phytohaemagglutinin-L (PHA) and staphylococcus enterotoxin B (SEB) or non-specific responses such as spontaneous secretion of cytokines in culture medium. Age and sex details of the study population are shown in Table 1. Inhaled steroids were used by 13/18 atopic asthmatics, 3/4 non-atopic asthmatics and 2/11 atopic non-asthmatics. With the exception of one control subject, none of the participants assumed oral steroids during the month prior to sampling. During the year before sampling oral steroids were used by 2/18 atopic asthmatics and 1/4 non-atopic asthmatics. Bronchodilators were used by 17/18 atopic asthmatics and 4/4 non-atopic asthmatics. The Research Ethics Committee at St. Mary's Hospital approved the study.

Skin prick test

Skin prick test (SPT) was performed using antigens manufactured by ALK Abelló (Hørsholm, Denmark). The panel included grass pollen, house dust mite, cladosporium, alternaria, aspergillus, birch pollen, plane tree pollen, nettle pollen, cat dander, dog dander and negative and positive (histamine) control. Responses of 3 mm or greater maximal weal diameter at 15 min after challenge were considered positive.

Table 1. Study population

Clinical classification	Mean age (range in years)	Males (n)	Females (n)	Total (n)
Atopic asthmatic	38 (20–59)	7	11	18
Non-atopic asthmatic	50 (35–68)	1	3	4
Atopic non-asthmatic	32* (22–45)	3	7	10
Non-atopic non-asthmatic	39 (19–79)	3	8	11
Total study population	38 (19–79)	14	29	43

*Based on nine subjects.

Only tests with negative responses to allergen diluent and positive responses to histamine were considered.

Antigens used in vitro

FG recombinant fusion protein comprising amino acids 1–526 of F (RSV SS2) and amino acids 69–298 of G (RSV A2) (patent WO 98/18819) was kindly provided by SmithKline Beecham Biologicals (Rixensart, Belgium). Tetanus toxoid was purchased from Calbiochem (San Diego, CA, USA). PHA and SEB were purchased from Sigma (Gillingham, UK). Der p (mite), Bet v (birch) and Fel d (cat) antigens were kindly donated by ALK Abelló. RSV A2 strain was grown in HEp-2 cells in the authors' laboratory and titrated by immunoplaque assay as described [13]. Human rhinovirus 16 (RV16) was prepared by passage in Ohio HeLa cells from virus donated by Elliott Dick (University of Wisconsin, Madison, WI, USA) and its identity confirmed by neutralization with specific antiserum (ATCC). TCID₅₀ was determined by titration for cytopathic effect in HeLa cells [14]. The stimuli used were: PHA (10 µg/mL), SEB (0.1 µg/mL), tetanus toxoid (1 µg/mL), recombinant FG fusion/attachment protein of RSV (1 µg/mL), Fel d (50 µg/mL), Der p (50 µg/mL), Bet v (50 µg/mL), RSV (m.o.i. = 5), human rhinovirus (HRV) (m.o.i. = 5), Hep2 cell mock-infected control for RSV, HeLa cell mock-infected control for HRV.

ELISPOT assays for IFN-γ and IL-4

Freshly separated PBMC were stimulated for 12 h in round bottom 96-well plates and then distributed into Millipore MAHA S45 10 plates which had been precoated overnight at 4–8 °C with sterile anti-human IFN-γ antibody 1-D1K or anti-human IL-4 clone 82.4 (both from Mabtech, Nacka, Sweden) at 15 µg/mL in carbonate/bicarbonate buffer pH 9.6 and blocked at 37 °C for 4–6 h with culture medium supplemented with serum. Each well received 5 × 10⁴ cells and 3 × 10⁵ cells for IFN-γ and IL-4 assays, respectively. After 48 h at 37 °C, the wells were washed extensively with phosphate buffered saline (PBS) and distilled water to remove all cells, then incubated at room temperature for 4 h with 1 µg/mL of biotinylated antibodies clone 7-B6-1 and clone 12.1 (anti-IFN-γ and IL-4, respectively, both from Mabtech). After further washes, 100 µL streptavidin-alkaline phosphatase conjugate (Sigma E-2636) diluted 1:1000 in PBS was added for 2 h; after further washes, 100 µL/well of BCIP/NBT substrate (Sigma B-5655) was added for 15 min and the reaction stopped by washing in tap water. Spots were counted with a dissection microscope at a magnification of 25 ×. Counts obtained by two independent observers were averaged. All frequencies are reported as cytokine spots per million PBMC, rounded to integers.

Statistical analysis

Statistical analysis was performed with Minitab statistical software version 12. Median cytokine ELISPOT responses were compared using the non-parametric Kruskal–Wallis test. *P*-values < 0.05 were considered significant.

Results

Skin prick test reactivity

The extent of sensitization of the study subjects to common allergens was assessed by skin prick test. As summarized in

Table 2, grass pollen reactivity was the most common, closely followed by house dust mite and cat allergens. The majority of subjects reacted to two or more allergens (data not shown).

Overall frequency of IFN- γ and IL-4 responses in the study population

The median frequency of IFN- γ -secreting cells was consistently higher than that of IL-4-producing cells, with the exception of wells stimulated with Der p which produced the highest median frequency of IL-4-producing cells (40 cells per million).

RSV-specific cells were found at the highest frequency of IFN- γ production (median: 440/million PBMC), followed by HRV (120/million PBMC) and FG (100/million PBMC). Median allergen-specific IFN- γ frequencies (Der p, Bet v and Fel d) were 20/million PBMC (Table 3).

Atopic subjects have higher frequencies of cells secreting IFN- γ in response to FG

When the responses were stratified by asthma and atopy the IFN- γ response to FG was significantly higher in atopics than in non-atopics (regardless of asthma) (140 vs. 20, $P = 0.04$) while no significant difference was seen between asthmatics and

non-asthmatics (regardless of atopy). Higher IFN- γ responses to RSV and HRV were observed in atopic compared to non-atopic individuals, but were not found to be statistically significant. IFN- γ responses to the allergens were not significantly different between groups (Table 4).

IL-4-secreting T cells responding to the cat allergen Fel d and birch pollen allergen Bet v are increased in adult asthmatics

The median frequency of IL-4-producing T cells specific for all allergens and viral antigens in our panel was higher in asthmatic adults when compared to non-asthmatic controls (Table 4). This difference was highly significant in responses to cat allergen Fel d (37 in asthmatics vs. seven in non-asthmatics, $P < 0.01$) (Fig. 1) and also significant for birch pollen Bet v (20 in asthmatics vs. three in non-asthmatics, $P < 0.04$). (Table 4). The median frequency of Fel d-specific IL-4 secretors in non-atopic asthmatics was very high (113, $P = 0.02$) but this group contained only four individuals. If these were removed, the comparison of atopic asthmatics vs. non-asthmatics remained significant (median 30 vs. seven, $P = 0.01$). Atopic status as defined by response to any allergen in the skin prick test was not correlated with significant differences in IL-4 responses to

Table 2. Skin prick test reactivity in the study population ($n = 43$)

	Positive*
Grass pollen	21
House dust mite†	19‡
Cat†	14‡
Dog	12
Plane tree mix	11
Birch pollen†	10§
Cladosporium	6
Alternaria	4
Aspergillus	3
Nettle	1

*Number of subjects with a SPT result of ≥ 3 mm. †These allergens were also tested in ELISPOT assays. ‡SPT not obtained for two subjects. §SPT not obtained for three subjects.

Table 3. IFN- γ and IL-4 responses in the study population ($n = 43$)

Stimulus	Response	Median*	Minimum	Maximum	Q1	Q3
FG	IFN- γ	100	0	4780	20	500
Fel d	IFN- γ	20	0	4340	0	180
Bet v	IFN- γ	20	0	4400	0	60
Der p	IFN- γ	20	0	600	0	40
RSV	IFN- γ	440	20	3360	120	1120
HRV	IFN- γ	120	0	1440	40	320
FG	IL-4	13	0	586	0	167
Fel d	IL-4	17	0	636	3	77
Bet v	IL-4	10	0	163	3	37
Der p	IL-4	40	0	796	7	160
RSV	IL-4	10	0	763	3	47
HRV	IL-4	13	0	513	3	53

*Numbers represent cytokine producing cells per million PBMC.

Table 4. Cytokine responses in asthmatic and atopic subjects

Cytokine	Stimulus	Non-atopics ($n = 15$)*	Atopics ($n = 28$)*	P	Non-asthmatic ($n = 21$)*	Asthmatic ($n = 22$)*	P
IFN- γ	FG	20 (0, 140)	140 (20, 825)	0.04	100 (20, 180)	80 (15, 885)	0.53
	RSV	140 (100, 880)	500 (265, 1180)	0.12	600 (110, 1210)	390 (215, 1015)	1.00
	HRV	80 (20, 160)	220 (40, 335)	0.10	120 (20, 240)	200 (40, 395)	0.30
	Fel d	0 (0, 160)	20 (0, 520)	0.34	20 (0, 170)	20 (0, 620)	0.55
	Bet v	20 (0, 400)	0 (0, 55)	0.45	0 (0, 50)	20 (0, 100)	0.63
	Der p	20 (0, 40)	20 (0, 40)	0.95	0 (0, 20)	20 (0, 60)	0.08
IL-4	FG	100 (0, 246)	13 (0, 149)	0.35	13 (0, 148)	25 (3, 241)	0.43
	RSV	10 (0, 53)	10 (3, 46)	0.75	7 (0, 43)	15 (6, 49)	0.24
	HRV	13 (3, 57)	15 (3, 49)	0.88	7 (2, 32)	17 (7, 62)	0.13
	Fel d	10 (3, 80)	17 (3, 72)	0.80	7 (2, 23)	37 (10, 163)	0.01
	Bet v	3 (0, 50)	10 (3, 35)	0.46	3 (0, 15)	20 (3, 54)	0.04
	Der p	30 (7, 160)	48 (8, 175)	0.79	17 (3, 172)	78 (10, 160)	0.23

*Numbers represent median frequencies of cytokine producing cells per million PBMC, followed by 2nd and 3rd quartile in brackets. Significant differences are in bold characters.

any of the antigens in the panel as measured in ELISPOT assays. For the recombinant chimeric FG the median frequency of IL-4 secreting T cells was 7.5 times higher in non-atopic individuals than in atopics, although the difference was not statistically significant (Table 4).

Lack of correlation between SPT and cytokine ELISPOT for individual antigens

Given the lack of correlation between generic atopic status defined by SPT and most cytokine responses, we checked whether IFN- γ and IL-4 responses to allergens Der p, Fel d and Bet v detectable by ELISPOT correlated with the classification of an individual as allergic to house dust mite, cat dander and birch pollen, respectively, on the basis of SPT reactivity. As shown in Table 5, we found no such correlation (Kruskal–Wallis test, adjusted for ties).

There was a non-significant increase in IL-4 spots in individuals with cat dander SPT ≥ 3 mm vs. < 3 mm (22 vs. 10, $P = 0.59$); birch pollen SPT ≥ 3 mm vs. < 3 mm (25 vs. 8, $P = 0.49$); and house dust mite SPT ≥ 3 mm vs. < 3 mm (73 vs. 48, $P = 0.38$).

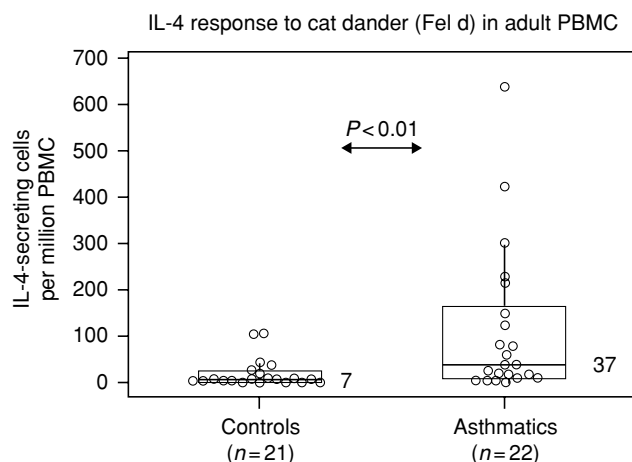


Fig. 1. Frequency of cat allergen-specific, IL-4-secreting T cells in adult asthmatics. PBMC from adult asthmatics and non-asthmatic controls were stimulated with Fel d (50 μ g/mL) for 2 days and IL-4-secreting cells measured by ELISPOT. Box plots show the median (indicated by the numbers) and 1st and 3rd quartiles. The whiskers indicate highest and lowest adjacent values. Values outside the whiskers are considered outliers.

Table 5. Comparison of skin prick tests and ELISPOT results

Allergen	SPT	N	IFN- γ		IL-4	
			Median*	P	Median*	P
House dust mite†	< 3 mm	22	10	0.11	48	0.38
	≥ 3 mm	19	20		73	
Cat dander†	< 3 mm	27	20	0.67	10	0.59
	≥ 3 mm	14	20		22	
Birch pollen‡	< 3 mm	30	20	0.60	8	0.49
	≥ 3 mm	10	0		25	

*Frequency of cytokine-producing cells per million PBMC. †SPT not obtained for two subjects. ‡SPT not obtained for three subjects.

Discussion

We found that adult asthmatics have a sixfold increase in the frequency of IL-4-secreting T cells specific for cat and birch allergens compared with non-asthmatics, but that atopy *per se* had no effect on the frequency of antigen-specific cytokine-producing cells. IFN- γ responses to allergens and viral antigens were largely similar in atopics and non-atopics and IL-4 responses to viral antigens did not differ between groups. An unanticipated finding was the significantly higher frequency of FG-specific IFN- γ secretors in atopic individuals.

These results complement our previous findings in 7.5-year-old children who had developed asthma after severe RSV bronchiolitis in infancy [8]. In that study we observed that IL-4-producing T cells specific for RSV antigens and cat allergen were more frequent in PBMC from ex-bronchiolitics (who had a cumulative prevalence of asthma of about 30%) when compared with sex-, age- and location-matched controls (with a cumulative asthma prevalence of about 3%) [7, 15]. Both cases and controls had similar IFN- γ responses to RSV and aeroallergens. We were surprised that such a difference could be detected several years after the bronchiolitic episode. Others have shown that children who had RSV lower respiratory tract illnesses before the age of 3 years had a significantly increased risk of wheeze by age 6 years, and this returned to background level by age 13 years [16]. RSV infection can be particularly severe in infants, often resulting in bronchiolitis, while it is generally mild in adults, who generally experience just a common cold. The differences in T cell responses to RSV between children and adults might be involved in this different clinical pattern.

Studies in adult asthmatics have questioned the simple hypothesis of a Th2/Th1 imbalance in asthma. After stimulation of adult PBMC with PMA and ionomycin, stronger IFN- γ responses (both in terms of overall secretion measured by ELISA and frequencies of IFN- γ^+ T cells) were shown in adult asthmatics [5, 6]. Others have found no difference in the frequencies of IL-4- and IFN- γ -expressing T cells in the PBMC of asthmatic adults, but higher expression of IFN- γ in the BAL of adult asthmatics [3]. In our work using human CD4 T cells clones specific for house dust mite peptide epitopes, we observed that polyclonal stimulation with PMA and ionomycin induces high IFN- γ and lower IL-4 and IL-5 expression while specific peptide stimulation induces high IL-4 and IL-5 and lower IFN- γ expression [17]. Studies of asthmatics need to be reviewed in the light of these results.

Our findings in this study confirm that a large subset of asthmatics has reactivity to aeroallergens (cat and birch) that is mediated by IL-4-secreting cells detectable in the circulation. Despite HDM reactivity being the most commonly detected by SPT, Der p responses detectable by ELISPOT showed only a non-significant increase in asthmatics, suggesting that sensitization to house dust mite is common but not always associated with asthma. Most atopic individuals had positive skin prick test responses to multiple allergens, some of which may be more related with the pathogenesis of asthma.

The role of respiratory viruses such as rhinovirus and RSV in asthma exacerbations is well established [18–20]. We found that T cell memory responses to viral antigens (RSV, FG and HRV) were easily detectable in adults and IFN- γ -secreting cells were

more frequent than IL-4-secreting cells. The frequencies of these effectors are broadly in agreement with the expected values derived from limiting dilution studies of pathogen-specific T cell responses. We did not see a significant difference between asthmatic and non-asthmatic individuals. Stratification of subjects on the basis of atopy (any SPT reactivity) showed a significantly higher frequency of FG-specific IFN- γ -producing cells in atopic subjects compared to non-atopic controls. Responders specific for whole RSV or HRV viruses were also more frequent in atopic subjects, but the difference was not statistically significant. The interpretation of this difference between atopic and non-atopic subjects is not easy. Our operational definition of atopic subject is based on SPT reactivity to at least one of a panel of common aeroallergens, but does not include testing of viral antigens. One possible interpretation could be that viral infections increase the likelihood of sensitization to aeroallergens [21]. Also, the intensity of antiviral responses might depend on the time elapsed from the last exposure to virus antigens. More studies are required to ascertain whether these differences for respiratory viruses are reproducible and meaningful.

We conclude that there is no significant difference in IL-4 responses to RSV in asthmatics and atopics and that adult asthmatics have a different pattern of cytokine responses to viral antigens than asthmatic children. We compared ELISPOTs with SPT results, which are routinely used to evaluate the atopic status of patients. SPT positivity indicates the presence of allergen-specific IgE bound to mast cells and basophils and capable of releasing histamine upon contact with allergen (i.e. sensitization). An ELISPOT measures the frequency of cells specific for a given antigen and capable of producing cytokines upon activation by peptide fragments of the antigen bound to MHC molecules. The involvement of IL-4-secreting Th2 cells is a requirement for isotype switching leading to IgE production. In addition, the site of response is different. We did not find a correlation between the two read-outs. This may be for a number of possible reasons: limitations of SPT are well appreciated and whilst we used a standardized technique for administering the test, it has been shown that the reactivity of the skin depends on age, sex, season and even time of the day making SPT a convenient albeit inaccurate estimator of allergic sensitization.

In addition, the commonly used cut-off value of 3 mm weal is considered by some to allow too many false positive results [22].

The correlation between RAST-determined levels of serum IgE specific for birch and cat allergens and IL-4 ELISPOT in selected cat or birch allergic individuals was described by Gabrielsson et al. [23] who also reported higher absolute frequencies of IL-4 spots than the present study. However that study employed a combination of suboptimal PHA and allergen to stimulate IL-4 production, while the frequencies reported in this study are based on stimulation by allergen alone.

In conclusion, the SPT and ELISPOT assays appear to measure different parameters of the allergic phenotype and are unrelated. However, patients with asthma showed increased numbers of cat and birch allergen-specific IL-4-producing cells in the peripheral blood. The frequency of FG-specific cells producing IFN- γ was higher in atopic individuals than in non-atopic controls, but other viral antigens or aeroallergens did not highlight significant differences.

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