Allergen-induced cytokine secretion in atopic and non-atopic asthmatic children

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Abstract

Background: Atopic asthma is characterised by excessive T helper 2 (Th2)-like immunity to allergens in the bronchial mucosa. The Th2-cytokine interleukin (IL)-4 induces IgE production, while the Th2-cytokine IL-5 promotes eosinophilic inflammation in the airways of asthmatics. Most asthmatics are atopic, but a sub-group is non-atopic. We hypothesise that allergen induced Th2-, particularly IL-5, responses, can be observed in peripheral blood in both atopic and non-atopic asthmatic children but not in healthy control children.

Aim: To determine IL-4, IL-5, IL-9, IL-10, IL-13 and IFN-γ secretion induced from PBMC by a broad panel of inhalant allergens (timothy, cat, birch, dog and house dust mite) in asthmatic children with and without sensitisation.

Materials and methods: The study included 13 atopic asthmatic, 5 non-atopic asthmatic, and 12 non-atopic non-asthmatic children. PBMC were stimulated with allergens and cytokine production was measured with ELISA.

Results: High levels of cat and dog induced IL-5 were more commonly observed in both atopic and non-atopic asthmatics than in controls. Children with atopic, but not non-atopic, asthma produced higher levels of allergen induced IL-4 and IL-9 than controls. Non-atopic asthmatics produced more IL-10 than atopic asthmatics after cat stimulation.

Conclusion: High levels of eosinophilia-associated IL-5 responses are induced by cat and dog allergen in both atopic and non-atopic asthmatic children. The Th2 cytokines IL-4 and IL-9 were associated only with atopic asthma, probably due to their IgE-inducing properties.

Keywords: asthma, atopy, allergen, childhood, cytokines, Th2

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Abbreviations - 3 -

ELISA   enzyme-linked immunosorbent assay

HDM   house dust mite

IFN   interferon

Ig   immunoglobulin

IL   interleukin

ISAAC international study of asthma and allergies in childhood

OD   optical densities

PBMC peripheral blood mononuclear cells

PBS   phosphate buffered saline

PHA   phytohaemagglutinin A

SPT   skin prick test

Th   T helper

Introduction

Atopic asthma is characterised by excessive T helper 2 (Th2)-like immunity to allergens in the bronchial mucosa [1], circulating specific immunoglobulin E (IgE) antibodies, positive skin prick tests (SPT) to common aeroallergens, and airway hyperresponsiveness [2]. Th2 cells produce immunoglobulin E (IgE) synthesis promoting interleukin-4 (IL-4) [3] and IL-13 [4], eosinophilia promoting IL-5 and IL-9 [5, 6], while interferon-γ (IFN-γ) from Th1 cells downregulate IgE synthesis [3]. Interleukin-9 also enhances IgE production [7] and stimulates T cell and mast cell growth [8, 9]. Interleukin-10 is an anti-inflammatory cytokine [10], that inhibits both Th1 and Th2 cells and IgE synthesis [11], as well as shortens eosinophil survival [10].

The immunological mechanisms occurring in the subgroup of patients with intrinsic (non-atopic) asthma, where no sensitisation can be demonstrated, are less well characterised, particularly in children. In a series of bronchial biopsy studies of asthmatic adults, both atopic and non-atopic asthma were characterised by infiltration of eosinophils, and expression of IL-4 and IL-5, eosinophil chemotactic cytokines and receptors [12-14]. It has been suggested that non-atopic asthmatics are allergic to an as yet undetected allergen or that IgE production in non-atopic asthmatics is local rather than generalized [14]. It is possible that allergen specific Th2 cells have been activated and perform their effector functions locally in the lung. Those T cells may then be identified in the circulation of non-atopic asthmatics. If allergens induce inflammation also in

the lungs of non-atopic asthmatics, these patients could benefit from immunotherapy and allergen avoidance [14].

Others have found that IL-5 protein levels in bronchoalveolar lavage are elevated both in atopic and non-atopic asthmatic adults, as compared to controls, whereas IL-4 is detected only in atopic asthmatics [15]. As IL-4 and IL-5 are differentially regulated [16-18], eosinophil mediated inflammation in the absence of IgE may be possible. We hypothesised that high levels of allergen induced Th2-, particularly IL-5, responses can be observed in peripheral blood of asthmatic children, independent of sensitisation, but not in healthy children.

The aim of this study was to determine IL-4, IL-5, IL-9, IL-10, IL-13 and IFN-γ secretion induced from peripheral blood mononuclear cells (PBMC) by a broad panel of inhalant allergens (timothy, cat, birch, dog, and house dust mite) in atopic and non-atopic asthmatic children.

Materials and methods

Subjects

The study included 30 children, aged 13-15-years, identified from phase 2 of the Linköping part of the International Study of Asthma and Allergies in Childhood (ISAAC). Atopic symptoms were determined by the ISAAC phase 2 questionnaire. Asthma was defined as bronchial obstruction ≥ four times/year and a positive bronchial provocation test. Based on these criteria, 18 children were defined as asthmatics. To perform the bronchial provocation test, the baseline and post-challenge FEV_1 were recorded using a MasterScope spirometer (JAEGER, Hoechberg, Germany), as described in [19]. Briefly, at least two baseline spirograms were performed and the highest of two reproducible (within 5%) measures of FEV_1 was recorded as the baseline FEV_1. The children then inhaled hyperosmolar saline (4.5%). The time of inhalation was progressively increased, starting with 30 seconds and with a maximum total inhalation period of 15.5 minutes. After every inhalation, FEV_1 was measured and the fall in FEV_1 was calculated. The challenge test was stopped and considered to be positive, when the decline in FEV_1 was more than 15%.

At the time of blood sampling, skin prick tests (SPT) were performed in duplicate on the volar aspects of the forearms with standardised birch, timothy, cat, dog and house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) extracts (Soluprick®, strength 10 histamine equivalents in prick testing for all allergens, ALK, Hørsholm, Denmark).

Histamine hydrochloride (10 mg/ml) was used as a positive control and albumin diluent as negative control. The test was regarded as positive when the mean diameter was $\geq$ 3 mm. Thirteen of the asthmatic children were SPT positive to at least two allergens. Eleven children were SPT positive to timothy, 10 to birch, 12 to dog, 11 to cat and 3 to house dust mite. Five of the asthmatic children were SPT negative. Twelve non-atopic children who did not have asthma or other atopic conditions were included as controls.

Circulating IgE to birch, timothy, cat, dog and *Dermatophagoides pteronyssinus* was analysed with UniCAP™, according to recommendations of the manufacturer (Pharmacia Diagnostics, Uppsala, Sweden). Samples with IgE levels above 0.35 kU_A/ml were regarded positive. The thirteen SPT positive children all had circulating IgE to one or several of the allergens, whereas none of the SPT negative children had detectable circulating IgE to the allergens tested.

Ethics

The study was approved by the Regional Ethics Committee for Human Research at the University Hospital of Linköping.

Cell culture

Venous blood was drawn into heparin treated tubes (Vacuette, Greiner Labor technik, Kremsmünster, Austria). PBMC were isolated on Ficoll Paque density

gradient (Pharmacia Biotech, Sollentuna, Sweden) as described in detail elsewhere [20].

Aliquots of 1 ml, containing 1×10^6 cells, were cultured in AIM-V serum free medium (Life Technologies AB, Täby, Sweden) with 20 μM mercaptoethanol (Sigma-Aldrich, Stockholm, Sweden). Optimal allergen doses and time points for cytokine secretion were determined in a pilot study including cells from six adults. Doses tested were 1 000, 10 000 and 50 000 standardised units/ml and time points tested were 24, 48, 72, 96, 144 and 192 h. The cells were stimulated with birch, timothy, cat, dog and *Dermatophagoides pteronyssinus* allergen extracts from ALK at 10 000 standardised units/ml. Separate cultures were performed with 2 μg/ml monoclonal antibodies to the IL-4 receptor (R&D Systems, Abingdon, UK), enabling measurement of IL-4 [21]. Two μg/ml of phytohaemagglutinin A (PHA) was used as a positive control. Background responses (cultures with medium only) were subtracted from the allergen and PHA induced responses. The cells were cultured at 37°C with 5% CO_2 (Forma CO_2-incubator model 3862, Forma Scientific Inc., Marietta, Ohio, USA) for 144 h. The supernatants were collected after centrifugation and stored at –70°C.

Cytokine analyses

The levels of IFN-γ, IL-4, IL-5, IL-9, IL-10 and IL-13 were determined by Enzyme-Linked Immunosorbent Assay (ELISA). Anti-human IL-9 monoclonal antibodies MH9A4 (IgG2b) and MH9A3 (IgG1) were derived from C57Bl/6 mice immunised with human IL-9 coupled to ovalbumin, as described

previously for mouse IL-9 [9]. The MH9A3 antibodies were biotinylated using an EZ-Link™ Sulfo-NHS-LC-biotinylation kit (Pierce Chemical Co, Rockford, IL, USA), according to the protocol of the manufacturer. Costar 3690 plates (Corning Inc, Corning, NY, USA) were coated overnight at room temperature. The following mouse anti-human coating antibodies were used, diluted in carbonate buffer, pH 9.6; IL-4 and IFN-γ (clone no 3010.211 and 2571.811, respectively, R&D Systems, Abingdon, UK), IL-5 (clone no TRFK 5, PharMingen, San Diego, CA, USA), IL-9 (clone no MH9A4), IL-10 and IL-13 (coating antibody, PeliPair™ reagent set, CLB, Amsterdam, The Netherlands). The concentrations used were 2 μg/ml for IFN-γ, 4 μg/ml for IL-4, 0.25 μg/ml for IL-5, 5 μg/ml for IL-9, and a 1:100 dilution for IL-10 and IL-13. Excess free antibodies were washed off (Wellwash Ascent, Labsystems, Stockholm, Sweden) with 4x250 μl PBS-Tween (0.05% Tween 20 in PBS, pH 7.4), and the wells were incubated for 1 h at room temperature on a plate shaker (Thermostar, Labvision, Stockholm, Sweden) with 100 μl/well of low-fat milk to block non-specific protein binding sites.

The plates were washed as described above and 50 μl of samples or standards; recombinant human IFN-γ, IL-4 and IL-9 (R&D Systems), IL-5 (PharMingen), IL-10 and IL-13 (CLB) were added to duplicate wells. A control using AIM-V serum free medium with 20 μM mercaptoethanol was also included. The plates were incubated and washed as before.

Biotinylated detection antibodies, goat anti-human IFN-γ and IL-4 (R&D Systems), rat anti-human IL-5 (clone no JES1-5A10, PharMingen), mouse anti-

human IL-9 (clone no MH9A4), IL-10 and IL-13 (CLB), were then added (50 µl/well). All were diluted in high performance ELISA-dilution buffer (CLB), to the final concentrations of 0.20 µg/ml for IFN-γ, 0.50 µg/ml for IL-4, 1 µg/ml for IL-5 and 90 ng/ml for IL-9. The antibodies to IL-10 and IL-13 were diluted 1:100. This was followed by another incubation and wash.

Streptavidin-poly horseradish peroxidase (50 µl/well, 0.1 µM, CLB) was added, and the plates were incubated as before, but for 30 minutes, and then washed. Thereafter, 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich) was added (50 µl/well), and incubated as before but for 30 minutes in the dark. 1.8 M H_2SO_4 (50 µl/well) was finally added to stop the reaction. The amount of substrate converted to product was thereafter detected as optical densities (OD) at 450 nm in an Anthos ht II ELISA reader (Anthos ht II, Labdesign, Täby, Sweden). Values were expressed as pg/ml deduced from the OD of the standard curve after subtracting the blanks. The sensitivity limits for quantitative determinations were 2 pg/ml for IL-10 and IL-13, 3 pg/ml for IL-5, 6 pg/ml for IL-4,10 pg/ml for IL-9 and 25 pg/ml for IFN-γ .

Statistics

As the cytokine levels were not normally distributed, even after logarithmic transformation, non-parametric tests, corrected for ties, were used. Comparisons between unpaired groups were analysed with Mann-Whitney U-test. The chi-square test was employed for categorical variables, and Fisher's exact test was used when the expected frequency for any cell was less than five.

A p-value of <0.05 was considered as statistically significant. Calculations were performed with a statistical package, StatView 5.0 for Macintosh (Acabus Concepts Inc., Berkeley, CA, USA).

Results

Detectable levels of IL-4 were more commonly observed in atopic asthmatics than in controls after dog and birch stimulation, with a similar trend after cat stimulation (Table 1). Furthermore, the cat, dog and birch induced levels of IL-4 were significantly higher in atopic asthmatics than in controls, with a similar trend after stimulation with timothy.

The IL-5 secretion was significantly higher in atopic asthmatics than in controls after stimulation with cat, dog and HDM. Furthermore, non-atopic asthmatics also tended to produce more IL-5 than controls after cat and dog stimulation (Table 2 and Fig 1 a and b). Moreover, six atopic asthmatics, three non-atopic asthmatics but none of the control children had IL-5 levels above the median (of the whole group of children), both after cat and dog stimulation, whereas three atopic asthmatics, one non-atopic asthmatic and six control children had IL-5 levels below the median both after cat and dog stimulation (Fisher's exact test, p=0.03 for atopic asthmatics vs controls and non-atopic asthmatics vs controls).

Interleukin-9 secretion was more commonly detected, and the levels were higher, in atopic asthmatics than in controls after dog stimulation (Table 3). A similar trend was observed after cat stimulation.

Atopic asthmatic and non-atopic asthmatic children produced similar amounts of IL-10, IL-13 and IFN-γ, as control children after allergen stimulation (data not

shown). The cat induced IL-10 secretion was significantly higher in non-atopic asthmatic than in atopic asthmatic children, however (Fig 2).

Discussion

Both non-atopic and atopic asthmatic children more often produced detectable levels of IL-5 after cat and dog allergen stimulation than control children. Our results support and extend previous studies in adults, showing that both atopic and non-atopic asthma are associated with high levels of IL-5 in bronchial biopsies [12, 14] and bronchoalveolar lavage [15]. Interleukin-5 causes allergic inflammation by its eosinophil activating properties [5]. High numbers of eosinophils are found in the bronchial submucosa [13] and bronchoalveolar lavage [15] of both atopic and non-atopic asthmatics. Our results support the hypothesis that non-atopic asthma is caused by an allergen-induced inflammation with eosinophils without the production of IgE. As IL-4 and IL-5 are differentially regulated [16-18], eosinophil mediated inflammation in the absence of IgE may be possible. Although the size of the group of non-atopic asthmatic children is small, and we cannot exclude that the lack of differences in the levels of other Th2 cytokines than IL-5 after allergen stimulation is due to a type II error, our findings suggest that IL-5 is the cytokine most strongly connected to non-atopic asthma. As immunotherapy reduces IL-5 expression [22], it is tempting to speculate that also non-atopic asthmatic children could benefit from such treatment. That high levels of IL-5 were noted after cat and dog allergen stimulation, but not after stimulation with house dust mite or seasonal allergens, could be due to the high and continuous exposure to cat and dog allergens in Sweden [23]. Due to the Swedish climate, house dust mite allergen exposure is low [23].

Children with atopic asthma, but not non-atopic asthma, produced higher levels of IL-4 after allergen stimulation than control children did. These results are consistent with several other studies, where this IgE-inducing cytokine was strongly connected with sensitisation [24-28].

Children with atopic asthma also produced high levels of IL-9. This cytokine has IgE-inducing properties and also stimulates T-cell, mast cell and eosinophil proliferation [6-9]. Increased IL-9 mRNA expression [26] and secretion [28] after allergen stimulation have previously been associated with atopic disease. The similar IL-13 secretion in the different groups of children may be due to the fact that IL-13 expression is less tightly regulated than IL-4 production, and not restricted to Th2 cells [29].

Interleukin-10 reduces IgE production, prevents eosinophil survival and has anti-inflammatory properties [10]. In this study non-atopic asthmatics produced more IL-10 than atopic asthmatics after cat stimulation. An inverse relationship between skin prick test positivity and IL-10 production has previously been reported in children with atopic symptoms [26, 28].

In conclusion, high levels of eosinophilia-associated IL-5 responses are induced by cat and dog allergen in both atopic and non-atopic asthmatic children. The Th2 cytokines IL-4 and IL-9 were associated only with atopic asthma, probably due to their IgE-inducing properties.

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Table 1. Interleukin-4 secretion after allergen stimulation (**frequency,** *i e* **(%) of samples with detectable levels and median (range) (pg/ml) in samples with detectable levels**).

The proportion of children with detectable IL-4 secretion among the atopic and non-atopic asthmatics was compared with the proportion in the controls with Fisher's exact test. The IL-4 levels in atopic and non-atopic asthmatics were compared with the levels in controls with Mann-Whitney *U*-test. ^{o}p=0.07, *p<0.05, **p<0.01.

Diagnosis	Cat	Dog	Birch	Timothy	HDM
Atopic asthma n=13	46%^{o}	46%*	31*	62%	8%
	10 (7-18)*	7 (6-9)**	10 (6-18)*	9 (7-95)^{o}	7
Non-atopic asthma n=5	0%	0%	20%	20%	0%
			9	8	
Controls n=12	8%	0%	0%	17%	8%
	7			8 (7-9)	8

Table 2. Interleukin-5 secretion after allergen stimulation (**frequency,** *i e* **(%) of samples with detectable levels and median (range) (pg/ml) in samples with detectable levels**).

The proportion of children with detectable IL-5 secretion among the atopic and non-atopic asthmatics was compared with the proportion in the controls with Fisher's exact test. The IL-5 levels in atopic and non-atopic asthmatics were compared with the levels in controls with Mann-Whitney *U*-test. $^{\circ}p=0.06$, $^{*}p<0.05$.

Diagnosis	Cat	Dog	Birch	Timothy	HDM
Atopic asthma n=13	100%	92%	31%	69%	54%
	25 (6-144)*	16 (7-37)*	19 (7-218)	14 (4-645)	10 (3-56)*
Non-atopic asthma n=5	100%	100%	20%	20%	40%
	22 (8-72)°	28 (5-78)°	32	93	16 (4-29)
Controls n=12	75%	67%	17%	33%	17%
	14 (3-28)	13 (5-24)	15 (5-25)	19 (4-43)	7 (5-9)

Table 3. Interleukin-9 secretion after allergen stimulation (**frequency, *i e* (%) of samples with detectable levels and median (range) (pg/ml) in samples with detectable levels**).

The proportion of children with detectable IL-9 secretion among the atopic and non-atopic asthmatics was compared with the proportion in the controls with Fisher's exact test. The IL-9 levels in atopic and non-atopic asthmatics were compared with the levels in controls with Mann-Whitney *U*-test. °$p=0.08$, *$p<0.05$.

Diagnosis	Cat	Dog	Birch	Timothy	HDM
Atopic asthma n=13	69%	69%*	8%	15%	54%
	122 (70-264)°	264 (99-726)*	31	160 (158-161)	84 (31-164)
Non-atopic asthma n=5	80%	60%	20%	20%	20%
	98 (34-192)	114 (61-145)	149	248	97
Controls n=12	50%	25%	0%	17%	8%
	61 (27-195)	13 (5-24)		74 (70-77)	118

Legends to figures

Fig 1. Cat (a) and dog (b) induced IL-5 secretion (pg/ml) in atopic asthmatic, non-atopic asthmatic and control children. $1x10^6$ PBMC/ml were cultured for 144 h with cat (a) and dog (b) allergen extract in AIM-V serum free medium supplemented with 20 μM mercaptoethanol. Interleukin-5 levels in the supernatants were measured with ELISA, and background responses (cultures with medium only) were subtracted. The lines indicate median values. Filled circles indicate sensitisation to cat (a) and dog (b). The IL-5 levels in atopic and non-atopic asthmatic children were compared with the levels in control children with Mann-Whitney *U*-test.

Fig 2. Cat (induced IL-10 secretion (pg/ml) in atopic asthmatic, non-atopic asthmatic and control children. $1x10^6$ PBMC/ml were cultured for 144 h with cat allergen extract in AIM-V serum free medium supplemented with 20 μM mercaptoethanol. Interleukin-10 levels in the supernatants were measured with ELISA, and background responses (cultures with medium only) were subtracted. The lines indicate median values. Filled circles indicate sensitisation to cat. The IL-10 levels in atopic and non-atopic asthmatic children were compared with Mann-Whitney *U*-test.
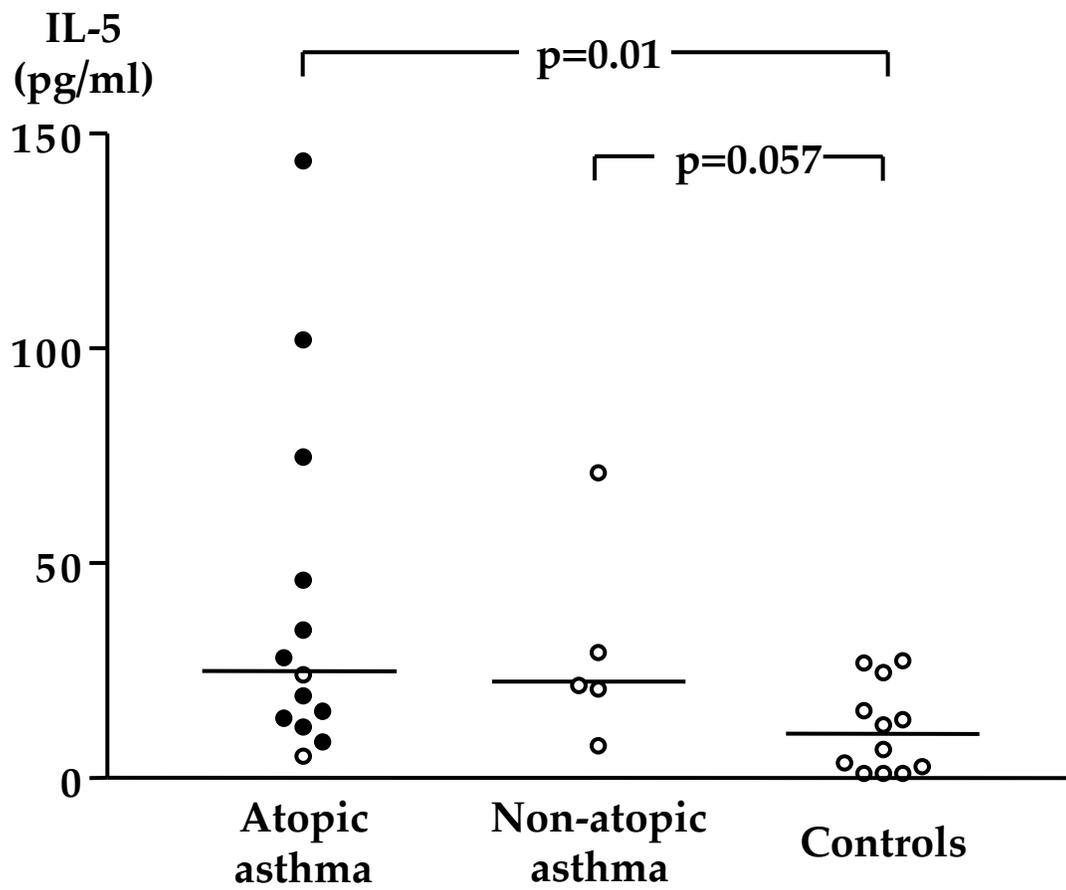
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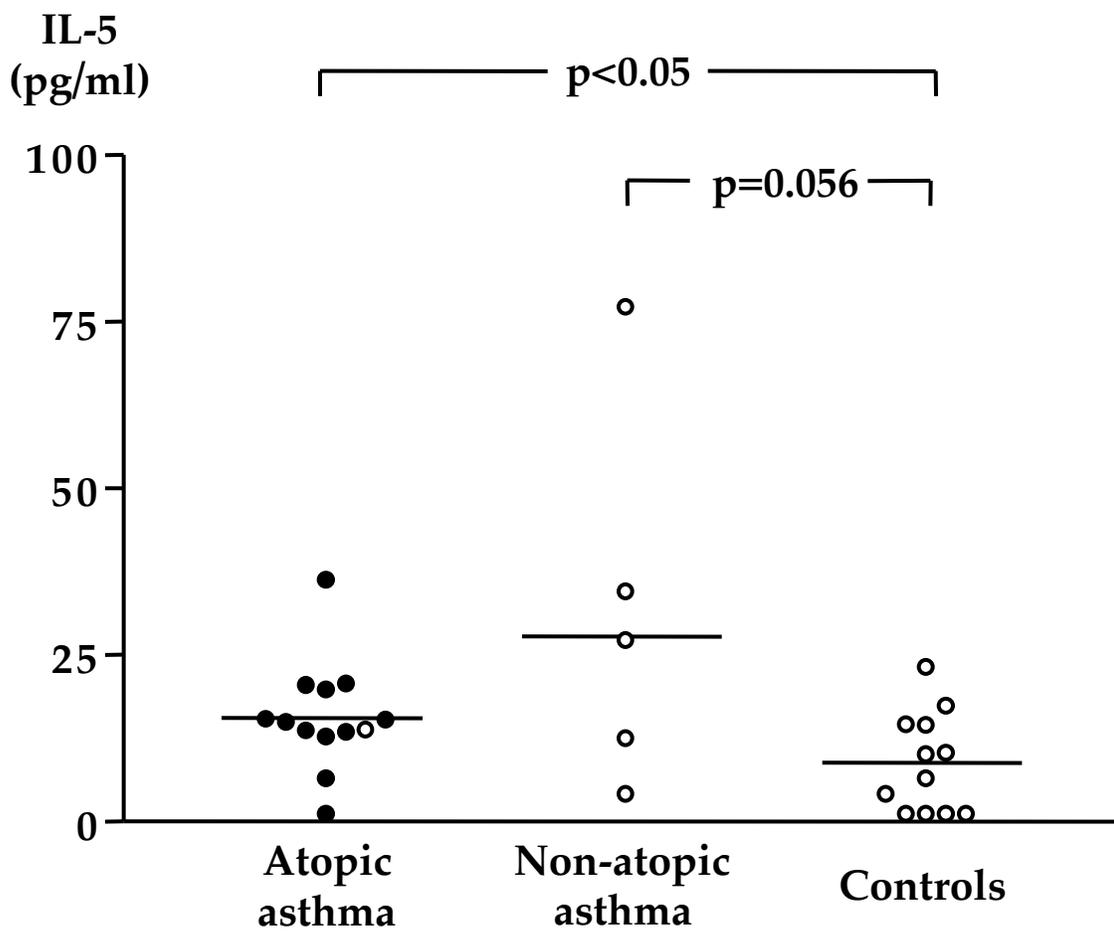
Fig 1

a)

b)

Fig 2