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Cytokine responses of peripheral blood mononuclear cells to allergen do not identify asthma or asthma phenotypes

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Condensed Title: Asthmatic PBMC cytokine responses to allergen

Key words: asthma, T cells, cytokines, allergen, eosinophils, phenotype

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Abstract

Background: Asthmatic patients are often differentiated based on their atopic status (atopic or nonatopic) and type of bronchitis (eosinophilic, neutrophilic, both, or neither). There is evidence supporting a central role for the T cell in asthma, but the role of allergen-induced T cell cytokines in driving disease in different asthma phenotypes remains unclear.

Objective: To investigate the hypothesis that peripheral blood mononuclear cells (PBMCs) from asthma patients with different phenotypes would react characteristically to a panel of common aeroallergens.

Methods: We incubated PBMCs from 41 asthma patients and 8 healthy controls with allergen and assessed PBMC proliferation by ³H-thymidine incorporation and the production of the cytokines IL-5, IL-17A, IL-23, IL-10, and IFN- γ by ELISA.

Results: No differences in PBMC proliferation or cytokine production were found in patients with asthma, compared with healthy controls, or between patients with different asthma phenotypes.

Conclusions & Clinical Relevance: PBMC cytokine responses to allergen are not able to assist in the discrimination between disease state, atopic status, or type of bronchitis in asthma.

Introduction

Asthma is an airway abnormality characterized by wide variations in airflow over short periods of time [1]. It is often accompanied by airway inflammation, reversible airway obstruction, and airway hyperresponsiveness leading to symptoms of

wheezing, difficulty breathing, coughing, and chest tightness [2]. Asthma can be classified as either atopic or nonatopic, depending on the allergic status of the patient [3], though symptoms and disease presentation are quite similar between the two groups [4]. In addition to this heterogeneity in phenotype, the nature of cellular infiltration of the airway and lumen may also vary from patient to patient and from time to time [5,6]. Cytokines such as IL-4, IL-5 and IL-13 play important role in recruiting eosinophils to the airway, while neutrophilic inflammation may be facilitated by Th-17 cytokines. Both types of cellular inflammation are regulated by T-lymphocytes.

T cells are a documented component of disease in atopic asthma. T lymphocytes in bronchoalveolar lavage from atopic asthmatic patients are activated and express increased IL-4 and IL-5 mRNA [7] and this expression correlates with airflow restriction, bronchial hyperresponsiveness and asthma severity scores [8]. In addition, regulatory CD25^{hi}FoxP3⁺ T cell numbers and function are reduced in bronchoalveolar lavage of children with asthma [9]. Nonatopic patients have also been shown to produce inflammatory responses to allergen. PBMCs from nonatopic asthmatic individuals can produce IL-5 in response to *Candida albicans*, and both inhaled and skin-prick *Candida* challenge can elicit a late phase response in these patients [10, 11]. Intradermal administration of peptides from the major cat allergen Fel d 1 (that do not cross-link IgE in vitro or cause an early wheal and flare cutaneous response) can provoke a late phase decline in FEV1 accompanied by chest tightness and wheezing [12].

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There is evidence in the literature to support an inflammatory role in asthma for the cytokines IL-5 [13-16], IL-17A [17-19], and IL-23 [20, 21]; as well as evidence to support a counter inflammatory or 'protective' role for IL-10 [17, 22, 23] and IFN- γ [24-26]. This study examined the ability of common allergens to drive the production of these cytokines in blood cells from asthmatic patients with varying phenotypes as well as healthy, nonasthmatic controls. Since allergen-specific T cells are present in peripheral blood and can be stimulated with their allergens to produce cytokines [27], we used PBMC analysis as a minimally invasive way to study patient sensitivity to allergen.

Our study had several aims. First, to assess the ability of allergen-induced PBMC cytokine production to distinguish: asthmatics from healthy controls, atopic patients from nonatopic patients, and between those patients experiencing eosinophilic and neutrophilic bronchitis. In so doing we attempted to evaluate allergen-induced cytokine production patterns as potential biomarkers of disease phenotype for the identification of subjects who may be suitable for selective cytokine blockade (e.g. with anti-IL-5). Second, to evaluate the association between allergen sensitivity (based on skin prick test results) and *in vitro* PBMC response to allergen. Third, to investigate the possibility that common allergens may act as previously undetected asthma triggers in nonatopic patients by stimulating Th2 and/or Th17 cytokine production in the absence of specific IgE (as detected by skin-prick test).

Methods

Patient Characteristics

Patients were recruited from the Firestone Institute for Respiratory Health at St. Joseph's Hospital in Hamilton, Ontario (see Table 1). They included 23 males and 18 females between the ages of 15 and 78. A diagnosis of asthma was based on a > 200 mL and $\geq 12\%$ improvement in FEV₁ after inhalation of salbutamol or a methacholine PC₂₀ of < 8 mg/mL. Atopy was defined as a positive skin-prick test to one or more allergens [28]. Asthmatics with an eosinophilic phenotype were defined as having $> 3\%$ sputum eosinophils on > 2 occasions [29]. A neutrophilic phenotype was defined as patients having normal sputum eosinophils with documented neutrophilia (total cell count $> 10 \times 10^6$ /g of which $\geq 65\%$ are neutrophils) on at least 2 occasions. 8 healthy controls were identified on the basis of PC₂₀ > 16 mg/mL, a negative skin-prick test, total serum IgE < 120 units/mL, and no history of asthma or allergic disease. This study was approved by the Research Ethics Board at St Joseph's Hospital in Hamilton, Ontario and all patients provided written informed consent.

Allergen Selection

Allergen extracts (Greer Labs; Lenoir, NC, USA) were plated in a 96-well plate at a concentration of 200 ug/mL in serum-free AIM-V media and frozen. Our panel included 32 allergens from the categories: mites and insects, animal epithelia and dander, fungi, and grass and weeds.

Mites and Insects: house dust mite (*D. farinae*, *D. pteronyssinus*, *E. maynei*), food/storage mites (*A. siro*, *L. destructor*), cockroach (*P. americana*, *B. germanica*).

Animal Dander and Epithelia: cat dander and epithelia, dog dander, hamster epithelia, mouse epithelia, rat epithelia.

Fungi: *Alternaria alternaria*, *Aspergillus fumigatus*, *Aureobasidium pullulans*, *Botrytis cinerea*, *Candida albicans*, *Cladosporium herbarum*, *Cladosporium sphaerospermum*, *Epidermophyton floccosum*, *Fusarium solani*, *Helminthosporium solani*, *Microsporum canis*, *Mucor circinelloides* f. *lusitanicus*, *Penicillium notatum*, *Phoma betae*, *Saccharomyces cerevisiae*, *Trichophyton rubrum*

Grass and Weeds: timothy grass, mugwort, ragweed.

Media was used as a negative control and Staphylococcal enterotoxin B (SEB) was used as a positive control. SEB is a superantigen that nonspecifically activates T cells.

Cell Culture

To isolate PBMCs, 25 mL of blood was layered onto 15 mL of Histopaque 1077 and centrifuged at 400 RCF for 30 minutes at 25°C with slow acceleration and deceleration. The buffy coat was removed and washed in 40 mL of serum-free AIM-V media at 250 RCF for 10 minutes at 25°C with maximum acceleration and deceleration. The cells were resuspended at 2.5×10^6 cells/mL in AIM-V. 100 μ L of

cells (2.5×10^5 cells) were added to each well of premade plates and incubated for 7 days at 37°C to create a final allergen concentration in the well of 100µg/ml. Media was not changed over the course of the experiment in order to avoid cytokine dilution.

Cytokine Measurement

At day 5, 100 µL of supernatant was removed from each well for cytokine analysis by ELISA (eBioscience; San Diego, CA): IL-5, IL-17A, IL-23, IL-10, and IFN-γ. ELISA limits of detection were as follows: IL-5: 4-500 pg/mL, IL-17A: 4-500 pg/mL, IL-23: 15-2000 pg/mL, IL-10: 2-300 pg/mL, IFN-γ: 4-500 pg/mL.

Proliferation Measurement

At day 6, PBMCs were pulsed with radioactive ^3H -thymidine (0.5 µCi per well) and incubated for 16 hours. PBMCs were then harvested onto glass fibre filter mats and assessed for proliferation (by way of ^3H -thymidine incorporation) using a MicroBeta counter. Proliferation results are displayed as a delta measurement, which indicates the degree of PBMC proliferation (in unit counts per minute) seen under each condition, compared with unstimulated cells. Counts per minute (CPM) values for media alone have been subtracted from the CPM values for each allergen well.

Statistics

Baseline characteristics were summarized using descriptive statistics. Differences between patient groups were analyzed using unpaired Mann-Whitney t tests. p

values < 0.05 are considered significant. Differences in cytokine production between steroid treatment groups were analyzed by one-way ANOVA. All analyses were performed using GraphPad software (La Jolla, CA).

Results

PBMC Responses in Asthma vs Healthy Controls

Our results show that exposure to allergen triggers both a proliferative and cytokine response from PBMCs when compared to no stimulation (media alone).

In response to allergen, PBMCs from asthmatic patients and healthy controls displayed similar proliferation. This suggests a comparable level of T cell activation in response to these antigens between the two groups (Figure 1).

PBMCs from asthmatic patients did not display significantly different levels of cytokine production from healthy controls. Although there was a trend for higher IL-5 production in asthmatic patients, it did not reach statistical significance. Healthy controls produced significantly more IL-10 and IFN- γ to the “Grass and Weeds” antigen class; this was due to the response to mugwort alone and may be broadly representative (Figure 2).

Differences in PBMC Responses Between Asthma Phenotypes

In asthma patients, neither allergen-induced PBMC proliferation or cytokine production was able to distinguish atopic status (Figure 3) or type of bronchitis (eosinophilic, neutrophilic, or neither) (Figure 4). T cells from atopic patients did

not respond more strongly to allergen than T cells from their nonatopic counterparts. Greater production of the pro-eosinophilic cytokine IL-5 was not seen in patients with a history of airway eosinophilia, and greater production of the pro-neutrophilic cytokines IL-17A and IL-23 was not seen in patients with a history of airway neutrophilia. Surprisingly, patients who had a history of asthma exacerbations characterized by lung eosinophilia produced significantly less IL-5 than those who did not. It is important to note that these eosinophilic patients continued regular steroid treatment for the control of their asthma during this study, and our data shows that overall IL-5 production declined with increasing steroid use (Figure 4D). This effect was not seen with proliferation or production of any other cytokines.

PBMC Responses to Known Sensitivities in Atopic Asthma

We investigated the relationship between PBMC responses and known IgE-mediated asthma triggers in the atopic asthmatic patient group. PBMC proliferation and inflammatory cytokine production did not consistently correspond with the patients' known sensitivities. Atopic patients produced Th2/Th17 cytokines to a variety of allergens, some of which were known to trigger their asthma, and some of which were not. They also failed to produce these cytokines in response to some of their known asthma triggers. Essentially, PBMC cytokine production patterns did not match patients' sensitization profiles. It should be noted that patients were not skin-prick tested to all the allergens on our experimental panel, but this lack of relationship between IgE-mediated sensitivity and cytokine production was true for

those allergens where skin-prick data exists. A representative patient example is provided in Figure 5.

PBMC Responses of Nonatopic Asthmatics to Allergen

We examined PBMC responses to allergen in nonatopic asthmatic patients in an attempt to pinpoint previously unidentified non-IgE-mediated, T cell sensitivities to allergen that may have been driving their disease. PBMCs from nonatopic asthmatics did produce Th2/Th17 cytokines in response to allergen, but based on the results from our atopic asthmatic group (indicating that cytokine production did not mirror known sensitivities), we cannot conclude that this may drive their asthma. A representative patient example is provided in Figure 6. PBMC responses were also not statistically different from healthy controls (data not shown).

Discussion

To our knowledge, this is the first study to examine PBMC responses to a broad panel of aeroallergens in a group of asthma patients that have been well characterized in terms of atopic status, allergic sensitizations and type of bronchitis. Our data indicate that cell-mediated responses to allergen, as measured by PBMC proliferation and cytokine production, do not distinguish asthma patients from healthy controls or differ between patients with different asthma phenotypes. It is not possible to determine a patient's disease state, atopic status, or type of bronchitis by examining their PBMC response to allergen. We also found that Th2 cytokine production did not mirror sensitization profile in atopic asthmatic patients.

There was no clear relationship between atopic asthmatics' known IgE-mediated allergen sensitivities and their PBMC response to allergen. No differences were seen when data was analyzed by grouping allergens into classes (i.e. fungi) or analyzed for individual allergens (i.e. *Candida albicans*).

These observations may be at odds with the currently accepted mechanism of allergic asthma: inhaled allergen cross-links antigen-specific IgE bound to FcεRI receptors on mast cells, effector T cells are recruited to the lung where they proliferate and secrete inflammatory cytokines, and eosinophils and/or neutrophils infiltrate the airway [3, 4, 30, 31]. Our results indicate that antigen-specific IgE does not necessarily correspond with markers of cell-driven inflammation, suggesting that a disconnect may exist between antigen-specific IgE and T cell activation and cytokine production.

T cell cytokines were chosen for investigation based on their potential ability to either drive asthma by instigating airway inflammation, or protect against airway disease by countering inappropriate immune responses. In a study by Hamid *et al*, IL-5 mRNA expression was increased in bronchial biopsies of asthmatics and was accompanied by a significant increase in activated eosinophils and T cells in the airway [13]. IL-5 has also been shown to correlate with symptom severity and hyperresponsiveness in asthma patients [14]. We recently demonstrated that administration of an anti-IL-5 antibody decreased asthma exacerbations and had a prednisone sparing effect in a group of severe asthmatic patients [16]. Molet *et al*

found increased IL-17A levels in the sputum and bronchoalveolar lavage of asthmatic patients [18], and levels of IL-17A in sputum have been shown to correlate with airway hyperresponsiveness to methacholine [32]. Elevated IL-17A has also been found in the plasma of allergic asthmatics [19] and correlated with plasma IL-23 levels. In animal models, IL-23 has been implicated in the recruitment of both eosinophils and neutrophils to the airway, the production of the Th2 cytokines IL-5 and IL-13, goblet cell hyperplasia, and airway hyperresponsiveness [33].

In contrast, IL-10 and IFN- γ have been reported to have a 'protective' role in asthma. IL-10 was the predominant cytokine produced by stimulated allergen-specific T cells from nonatopic donors (while their allergic counterparts produced IL-4) [17]. In a mouse model of allergic asthma, the adoptive transfer of allergen-specific CD4⁺CD25^{hi} T cells increased expression of IL-10 and reduced airway hyperresponsiveness and Th2 inflammation. These effects were ameliorated by the administration of an anti-IL-10 mAb [34]. In another model, administration of IFN- γ prevented the accumulation of T cells and eosinophils in the airway following antigen challenge in sensitized mice, while pretreatment with an anti-IFN- γ mAb had the opposite effect, increasing the infiltration of these cells into the airway [35]. Expression of the Th1 transcription factor T-bet has been found to be reduced in the airways of asthmatic patients [36], and serum levels of IFN- γ are significantly decreased in children with asthma [24].

Based on this evidence of an association of each of these cytokines with asthma and atopy, we were surprised to find a complete lack of differences between cytokine responses in our study patients with asthma (especially those in the atopic group) and healthy controls. Despite the strong role that allergen exposure is thought to play in driving asthma, and the central role that the T cell occupies in the mechanism of asthma pathogenesis, allergen-driven T cell cytokine production did not prove to be a strong biomarker of disease.

As patients maintained their normal asthma treatment during this study, we cannot discount the impact of steroid use on our results. We found that patients with a history of eosinophilic bronchitis produced significantly less IL-5 than their non-eosinophilic asthmatic counterparts, and this may be the result of elevated steroid use in the eosinophilic group. Other groups have found that glucocorticoids inhibit GATA-3 activity and IL-5 production in splenocyte cultures [23], and decrease eosinophil migration to the lung following allergen challenge [37]. Had the eosinophilic patients in our study not been taking high doses of steroids to control their asthma, a more significant role for PBMC-derived IL-5 as a biomarker of disease may have emerged. Patients were allowed to remain on corticosteroid treatment during the study since our goal was to examine asthmatic patients with persistent airway inflammation in spite of therapy and attempt to identify allergens driving this presently uncontrolled response.

This study was conducted using peripheral blood cells that may not be representative of the unique environment of the lung. It is possible that a

significantly stronger cytokine response to allergen occurs locally in the lungs of asthmatic patients, and that we are just unable to detect this in peripheral blood. There is evidence of a potential role for local IgE production in the asthmatic lung [30, 38], and a similar localized cytokine response may also occur. However, our results demonstrate that culturing a patient's PBMCs with allergen and measuring our selected cytokines would not identify potential asthma triggers.

Overall, our results indicate that allergen-induced proliferation and cytokine production in peripheral blood mononuclear cells is not a good biomarker of disease in asthma. Examining an individual's PBMC responses to allergen does not reveal their disease state, atopic status, allergic sensitizations, or type of bronchitis.

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- Accepted Article
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Table 1

	All patients (n = 49)	Asthmatics (n = 41)	Normal controls (n = 8)
Age (y)	46 (15)	48 (15)	36 (8)
Sex (% male)	51	56	25
Smokers (% smokers)	29	34	0
History of atopy (%)	33	39	0
Pre-bronchodilator FEV ₁ (L)	2.55 (1.0)	2.44 (1.1)	3.14 (0.44)
Pre-bronchodilator FEV ₁ (% predicted)	76.4 (21)	73 (21)	93.8 (10.2)
FEV ₁ /VC	0.72 (0.15)	0.68 (0.12)	0.92 (0.09)
Eosinophilic phenotype (n)	33	33	0
Neutrophilic phenotype (n)	6	6	0
ICS dose (median, IQR)	100 (1200)	1000 (1000)	0
Prednisone	5.8 (7.4)	6.9 (7.8)	0

Data provided as mean (SD) unless otherwise specified

Figure Legends

Figure 1.

Allergen-induced PBMC proliferation from asthmatic patients and healthy controls. Individual allergens are shown in (A) and allergens grouped by category are shown in (B). Data are expressed as mean delta CPM $\pm$ SEM. n = 39 (asthma) and n = 8 (control).

Figure 2.

Allergen-induced cytokine production from asthmatic patients and healthy controls. Data are expressed as mean $\pm$ SEM. n = 41 (asthma) and n = 8 (control).

Figure 3.

Allergen-induced PBMC responses in nonatopic and atopic asthma. Proliferation is shown in (A) and cytokine production in (B-F). Data are expressed as mean delta CPM $\pm$ SEM. n = 25 (nonatopic) and n = 16 (atopic).

Figure 4.

Allergen-induced cytokine production in different types of bronchitis. (A) IL-5 production in eosinophilic and noneosinophilic asthma. (B) IL-17A and (C) IL-23 production in neutrophilic and nonneutrophilic asthma. (D) Average allergen-induced IL-5 production from eosinophilic asthmatic patients compared across 5 steroid treatment groups. Data are expressed as mean $\pm$ SEM. n = 33 (eosinophilic), n = 9 (noneosinophilic), n = 6 (neutrophilic), n = 35 (nonneutrophilic).

Figure 5.

Allergen-induced cytokine production in a representative atopic asthmatic patient. This patient is sensitized to dust mite and shows dominant IL-5 and IL-17A production to several mites, but also to cat dander, mouse epithelium, and grass.

Figure 6.

Allergen-induced cytokine production in a representative nonatopic asthmatic patient.

Statement of Conflicts of Interest

In the past 3 years, Parameswaran Nair has received support for investigator-initiated research from GSK, Schering and Pulmatrix. He is listed on a global patent for a sputum filtration device and provides consultation to a University spin-off company, Cellometrics Inc.

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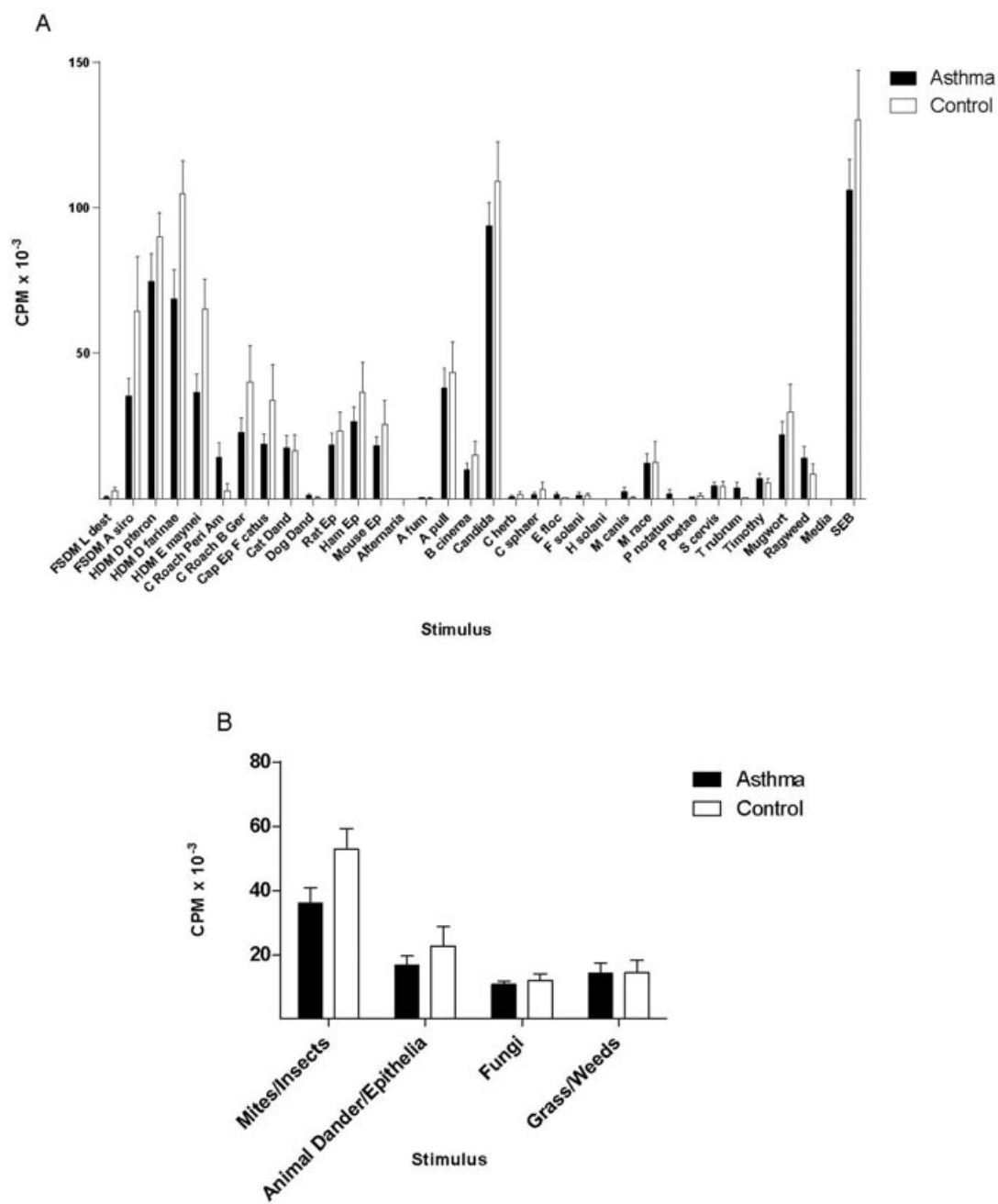


Figure 1

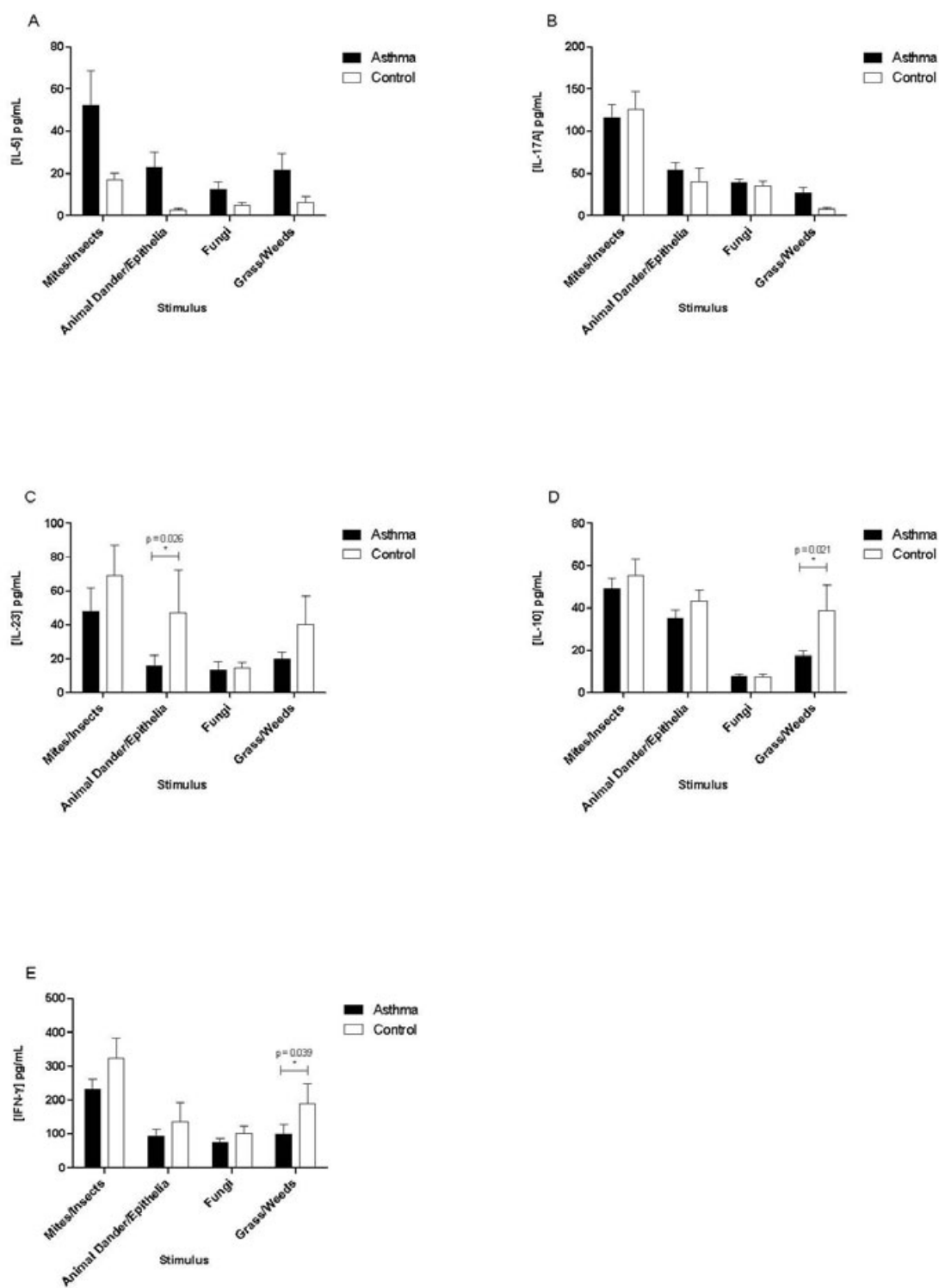


Figure 2

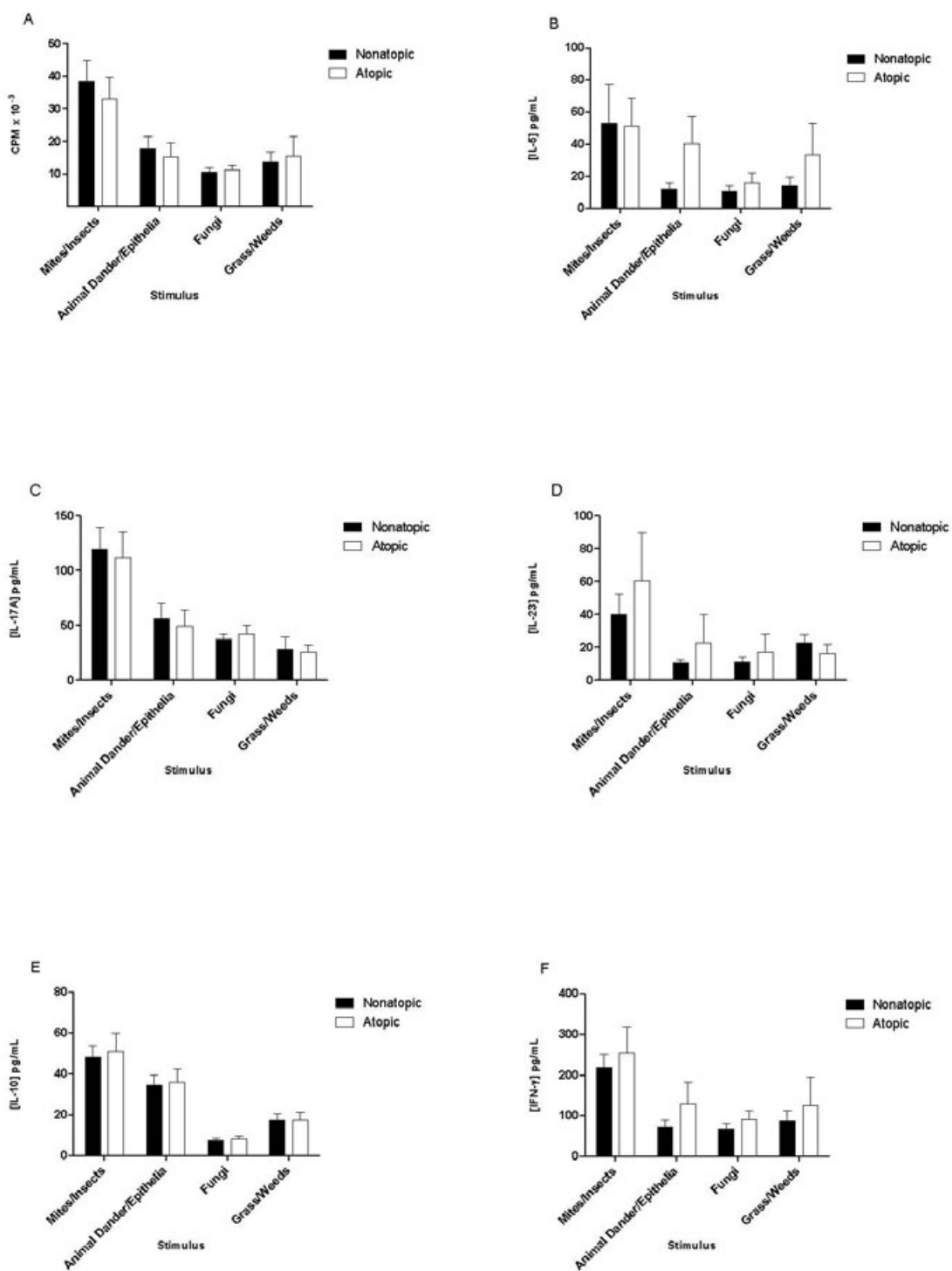


Figure 3

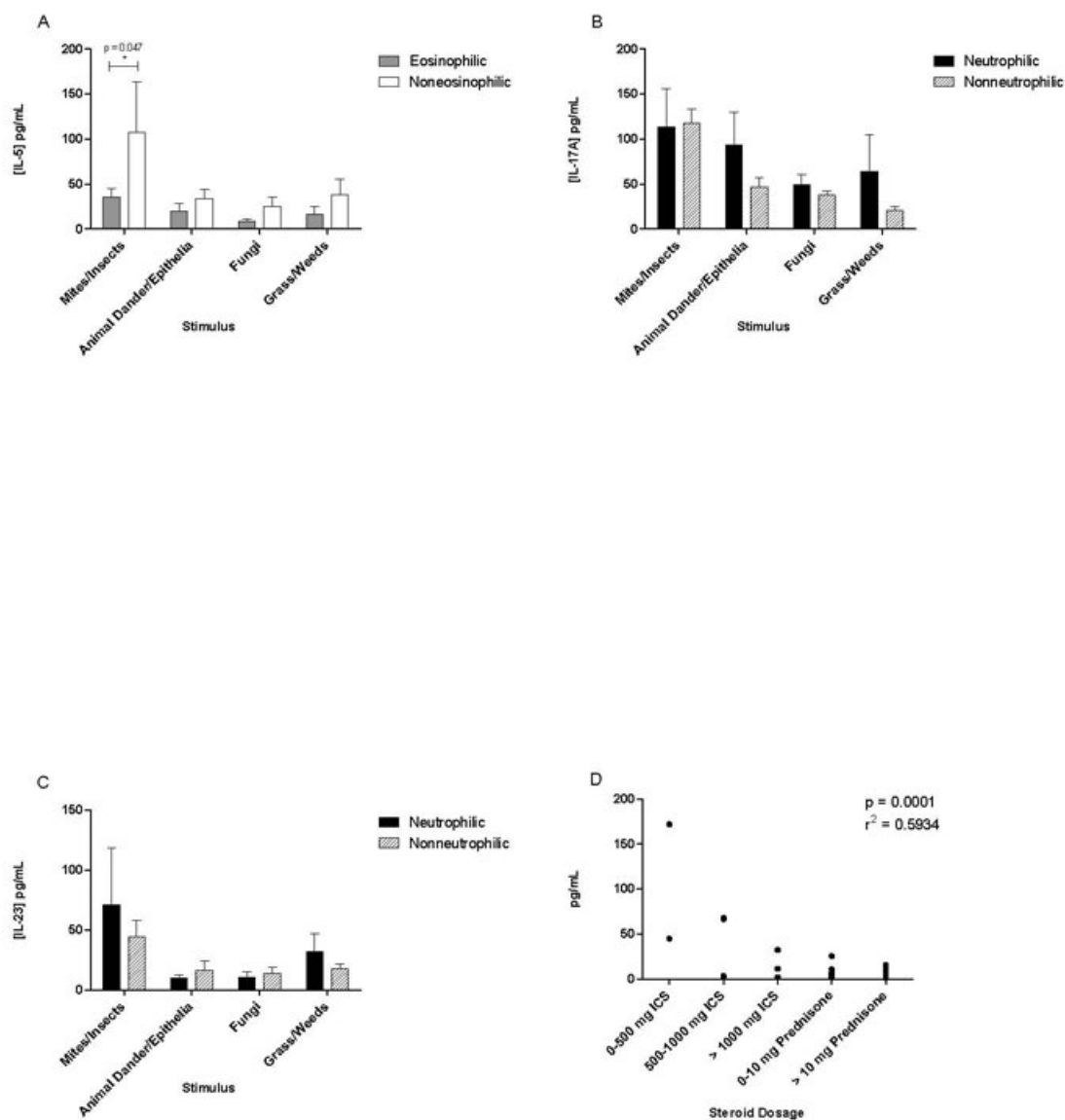


Figure 4

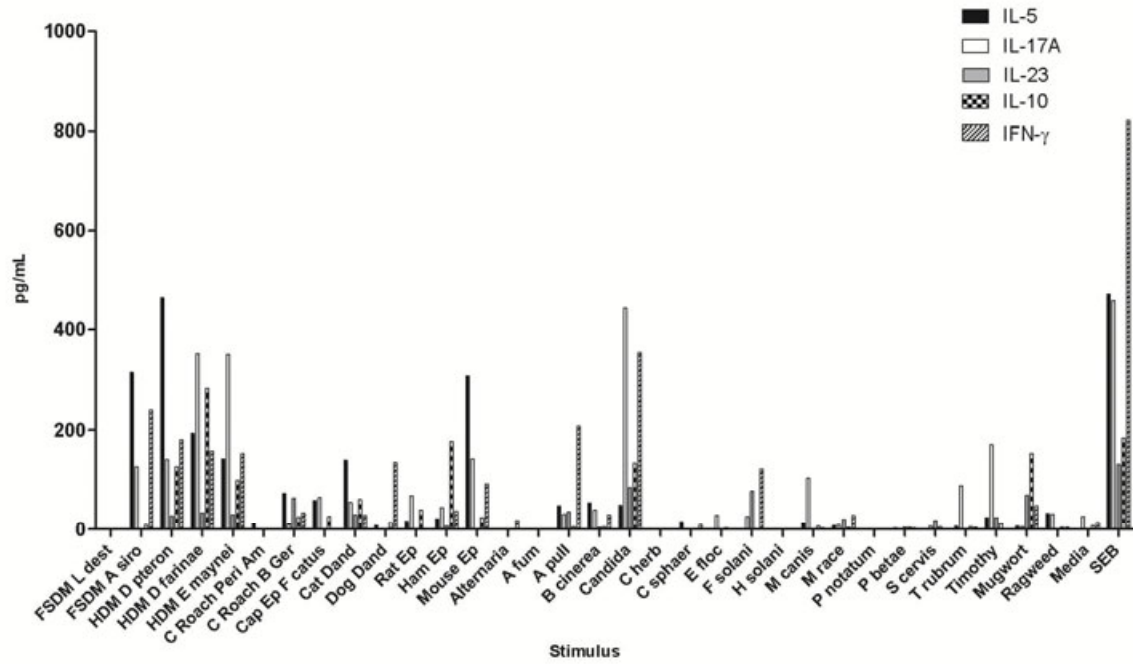


Figure 5

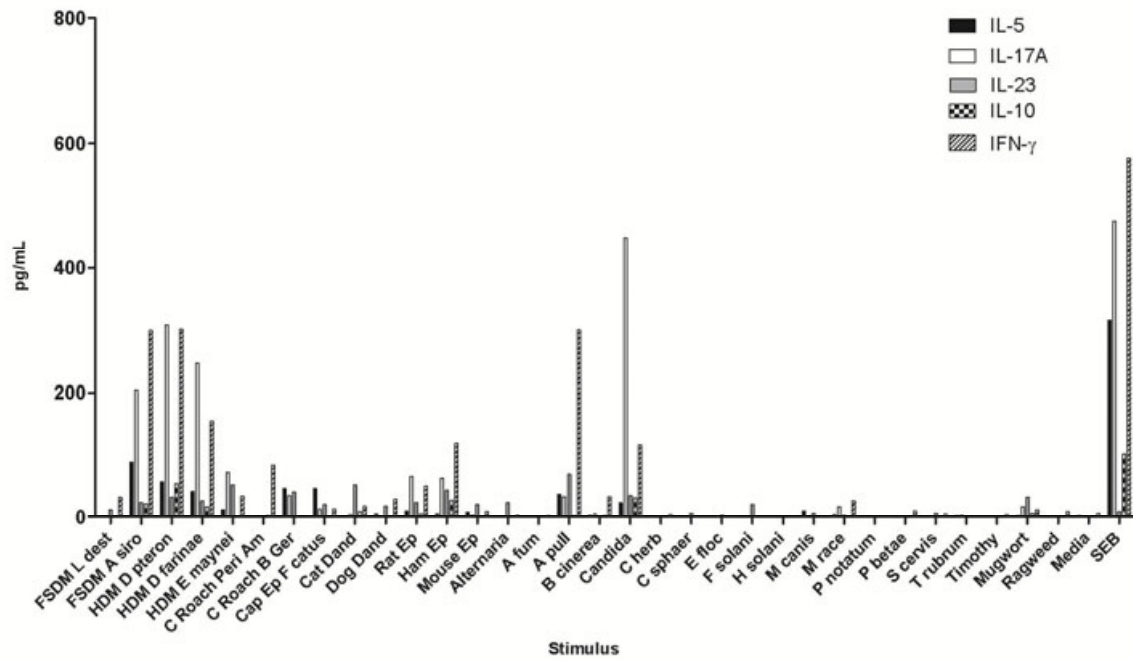


Figure 6