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**Increased IL-17A secreting CD4+ T cells, serum IL-17 levels, and exhaled nitric oxide
are correlated with childhood asthma severity**

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Background: Measuring fractional exhaled nitric oxide (FeNO) is a simple and noninvasive method for assessing airway inflammation. IL-17 plays an important role in T cell-dependent inflammatory response that occurs in allergic asthma, it could act as a potent activator of inducible nitric oxide synthase (iNOS) to amplify FeNO levels.

Objectives: To evaluate the differences in the CD4⁺IL-17A⁺ T cell counts, serum IL-17 levels, and FeNO levels in children with mild intermittent to moderate to severe persistent asthma classified by using the Global Initiative for Asthma (GINA).

Methods: One hundred twenty asthmatic children divided into the mild intermittent (n=42), mild persistent (n=42), and moderate to severe persistent (n= 36) groups and 20 healthy controls were recruited for the study. Information obtained at visits included the assessment of asthma severity according to GINA guidelines and C-ACT, lung function parameters, FeNO levels, CD4⁺IL-17A⁺ T cells counts from PBMCs, iNOS production by sputum cells and serum IL-17 levels.

Results: Serum IL-17 and FeNO levels were significantly higher in mild to severe persistent asthmatic patients than in intermittent asthmatics or healthy controls ($p < 0.05$). The percentage of CD4⁺IL-17A⁺ T cells was higher in moderate-to-severe persistent asthmatics than in mild asthmatics ($p < 0.01$). Moderate-to-severe asthmatics (n=5) exhibited greater iNOS production in sputum cells than mild cases (n=5). Decreased iNOS expression in sputum cells was noted in all subjects after IL-17 neutralizing antibody ($p < 0.05$). Serum

IL-17 levels were positively correlated with FeNO ($\rho = 0.74$; $p < 0.01$), negatively correlated with C-ACT ($\rho = -0.63$; $p < 0.01$) in asthmatics.

Conclusion and Clinical Relevance: CD4⁺IL-17A⁺ T cells counts and serum IL-17 levels in conjunction with augmented FeNO levels are systemic markers of childhood asthma, using these markers, prediction and potential therapeutics for persistent asthmatics may be developed.

Key words: C-ACT; IL-17; nitric oxide

ABBREVIATIONS

Childhood asthma control test	C-ACT
<i>Dermatophagoides pteronyssinus</i>	Der p
Fractional exhaled nitric oxide	FeNO
Forced expiratory volume in the first second	FEV1
Forced vital capacity	FVC
Global Initiative for Asthma	GINA
Interleukin-17	IL-17
Nitric oxide synthase	NOS
Maximal mid-expiratory flow	MMEF

INTRODUCTION

Th17 cells and the Th17 cell-derived cytokine IL-17A are involved in various autoimmune and chronic inflammatory diseases, including systemic lupus erythematosus, psoriasis, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and allergic asthma (1-6). Increased IL-17 and neutrophils levels are found in the sputum and the bronchoalveolar lavage fluid of patients with severe or corticosteroid-resistant asthma (7-9). IL-17 mediates the recruitment of neutrophils and can activate smooth muscle cells and epithelial cells in the airways to induce proinflammatory mediators (10-12). Although Th2 cells have traditionally been considered to be responsible for pathogenesis in allergy and asthma, Th17 cells may also play a key role in the airway inflammation. Th17 cells can upregulate Th2 cell-mediated eosinophilic airway inflammation and augment Th2 responses in asthma (13-16).

Spirometry is currently used to monitor asthma control, but this approach may not be informative about the degree of airway inflammation. Nitric oxide (NO) is a reactive, free radical gas that is produced by a variety of cells via activation of nitric oxide synthases (NOS) (17, 18). Measurement of the fractional exhaled NO (FeNO) has been suggested to be a

sensitive, noninvasive indicator for monitoring airway inflammation in children (19, 20). FeNO values are elevated in children with asthma at baseline and during acute exacerbations, and these levels are correlated with the magnitude of airway eosinophilia and bronchial hyperresponsiveness (21). Repeated FeNO measurements may provide guidance on how to adjust or to discontinue glucocorticoid administration, and monitoring FeNO protects asthmatics from excessive glucocorticosteroid intake (20, 22, 23).

The proinflammatory actions of IL-17 largely derive from inducing the expression of inducible nitric oxide synthase (iNOS) and the subsequent release of the cytotoxic and immunoregulatory free radical NO (24-26). The role of IL-17-induced NO in asthma has not been investigated to date, although it appears that the consequences of IL-17-mediated NO release, which results in airway inflammation, could be relevant for asthma outcomes.

In order to gain additional information on the roles of CD4⁺ IL-17A⁺ T cells and serum IL-17 in the severity of asthma, we investigated whether systemic inflammation markers, CD4⁺ IL-17A⁺ T cells and serum IL-17, and the airway inflammation biomarker FeNO, can reflect asthma severity and whether they were correlated with C-ACT scores. These findings may yield further evidence and provide a novel direction for immunotherapy in patients with persistent asthma.

Materials and methods

Subjects

One hundred twenty mildly intermittent to persistent asthmatic children (aged 5-18 years old) with sensitivity to house dust mites (Der p), demonstrated by a specific IgE test using the CAP system (Pharmacia Diagnostics, Uppsala, Sweden), were enrolled in this study. Asthma severity was evaluated on the basis of the revised Global Initiative for Asthma (GINA) criteria. All intermittent asthmatics (n=42) had episodic symptoms only (<1 per week), and none had night-time asthma. Mild persistent asthmatics (n=42) had daytime symptoms at least once a week, night-time asthma more than twice a month. Moderate (n=26) to severe (n=10) persistent asthmatics were grouped as moderate-to-severe asthmatics had day time symptoms on most days, night-time asthma more than once a week. Most of the moderate to severe asthmatic patients were treated by inhaled corticosteroids (ICS), and study participants were allowed to continue their usual ICS treatments. Twenty healthy subjects with normal serum IgE levels, who were negative for skin prick tests and specific allergen IgE by the CAP system, were selected as the healthy control subjects. All the patients enrolled into the study, in each group, completed the study. The study was performed with the approval of the Institutional Review Board (I050920) and all the subjects provided written informed consent by parents.

Childhood Asthma Control Test (C-ACT)

Symptoms were measured using the Childhood asthma control test scores (C-ACT). The C-ACT test is a seven-item questionnaire used to assess asthma control. Four of these items are answered by the child and three by the accompanying parents (27). C-ACT test and GINA guideline-based severity measure groupings were completed by a physician who was blinded to the results of FeNO or pulmonary function tests. Although the C-ACT test was developed for children between 4 and 11 years of age, we used it in all our study subjects to provide consistency when evaluating asthma control.

Measurement of the fractional exhaled nitric oxide (FeNO)

Pulmonary function parameters (Sensomedics, Yorba Linda, CA, USA) including FEV1, FEV1/FVC, MMEF, and PEFr were measured in all asthmatic patients. Values were expressed as a percentage of the predicted normal values for height and sex. Fractional exhaled nitric oxide levels were measured by electrochemical analysis with an NIOX MINO device (Aerocrine AB, Solna, Sweden). FeNO measurements were measured and blood sampling was taken at the same time without asthma acute exacerbation.

Measurement of CD4⁺ IL-17A⁺ T cells and CD4⁺ IL-4⁺ T cells

PBMCs were isolated using a Ficoll-Paque gradient centrifuge (Pharmacia, Uppsala, Sweden). A total of 1×10^6 cells were cultured in RPMI-1640 culture medium and divided into 96-well culture plates. PBMCs were stimulated with anti-CD3 mAb (2 μ g/ml) for 5 days for interleukin IL-17 analysis and *Dermatophagoides pteronyssinus* 2 (10 μ g/ml) (Indoor Biotechnologies, Cardiff, UK) for 5 days for interleukin IL-4 analysis, followed by stimulation of PMA (10 ng/ml) plus ionomycin (1 μ g/ml) for the last 5 hours, and brefeldin A (10 μ g/ml) was added for the final hour. Anti-human CD4, interleukin IL-17 mAb, interleukin IL-4 mAb and isotype-matched control mAbs conjugated with PE and PC5 (anti-human IgG1 PC5 conjugated mAb for CD4 & anti-human IgG1 PE conjugated mAb for IL-17A) were obtained from BD Biosciences (San Diego, CA). Intracellular cytokine staining for IL-17 and IL-4 was measured by using the standard procedures (eBioscience, San Diego, CA) (28), and analyzed by FACS using a scan flow-cytometer (FC500, Beckman Coulter, Fullerton, CA) by acquiring 10,000 events.

Enzyme-linked immunosorbent assay

In order to analyze the inflammatory cytokines in asthma, plasma IL-17 levels were measured using a Human IL-17 Quantikine ELISA kit (R& D Systems, Minneapolis, MN, USA).

Sputum induction and iNOS analysis

Sputum was induced by inhalation of 3% hypertonic saline after premedication with 400 µg of albuterol. Sputum plugs that appeared free salivary contamination were collected in a 50mL tube, wash and homogenized with Hanks' buffer (Gibco, NY) containing 0.1% dithiothreitol (Sigma, St. Louis, MO, USA). We centrifuged at 2000 rpm for 10 min at 4°C, removed supernatant, then incubated cell pellet with RPMI-1640 culture medium containing 10% fetal bovine serum (Gibco, NY). To test IL-17 as a potent activator of inducible nitric oxide synthase (iNOS) in allergic asthma, we measure iNOS expression in sputum cells (cell suspension was adjusted to 2×10^6 cells/mL) from different severity asthmatics treated with anti-IL17 neutralizing mAb (40ng/mL) (R&D, Minneapolis, MN) or medium alone for a day, washed cell pellet and lysis protein by cell-lysis buffer (Roche, Basel, Switzerland). We gauged protein levels of iNOS, beta-actin by Western blot analysis. Equal amounts of proteins in each study group were determined using a Bio-Rad protein assay kit (Bio Basic, CA), cellular proteins were resolved by 10% SDS-PAGE. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane, blocked with 5% nonfat milk in PBS/Tween 20 (0.1%), and probed with iNOS, beta-actin (GeneTex, Texas, USA). Following incubation with primary Abs for 1 h, the membrane was washed and incubated with HRP-conjugated anti-rabbit IgG Ab (GeneTex, Texas, USA) (1:10,000 in PBS/Tween and 1% BSA) and visualized using an ECL system (Millipore).

Statistical Analysis

All data are presented as mean $\pm$ SD. Differences of means compared for each group were analyzed using the Kruskal-Wallis test, followed by the Duncan test. Correlations were analyzed with Spearman's rank order correlation coefficient test. A level of $P < 0.05$ was considered significant.

Results

General characteristics of the subjects

The characteristics of the study subjects are summarized in Table 1. According to the GINA guidelines, the 120 patients with allergic asthma could be divided into three subgroups: 42 (35%) with mild intermittent asthma, 42 (35%) with mild persistent asthma, 36 (30 %) with moderate to severe persistent asthma. Age of participants was higher in the control group than three subgroups of patients with asthma (Table 1).

Patients with mild to severe persistent asthma had significantly increased total IgE ($p < 0.01$) and Der p-specific IgE levels ($p < 0.01$) compared to mild intermittent asthma patients (Table 1). Patients with moderate to severe persistent asthma had significantly increased total IgE compared to mild persistent asthma patients ($p = 0.02$) (Table 1). As shown in Table 1, C-ACT scores were significantly decreased in the mild to severe persistent asthma group as

compared to the intermittent asthma group ($p < 0.01$). Participants in the moderate to severe persistent asthma group showed remarkably decreased C-ACT scores, with values below 24 (Table 1).

FeNO and pulmonary function in asthmatic patients

When compared to healthy control individuals, FeNO levels were significantly higher in all asthmatics ($p < 0.01$) (Figure 1 a). FeNO levels were significantly increased in the mild to severe persistent asthma group compared to the mild intermittent asthma group ($p < 0.01$) (Figure 1a). There was a significant difference in the spirometry parameters, including the FEV1, FEV1/FVC, MMEF, and PEF values (expressed as percentages of the predicted values) in the moderate to severe persistent asthmatic subjects when compared to the mild intermittent to persistent asthmatic subjects ($p < 0.01$) (Table 1). However, unlike the differences that were observed for the FeNO levels, no differences were seen in the FEV1, FEV1/FVC, PEF, and MMEF values between mild intermittent and mild persistent asthmatic subjects ($p > 0.05$) (Table 1).

Serum IL-17 level in asthmatic patients

In order to study whether serum IL-17 levels were different among subgroups of asthmatic patients, we compared the IL-17 levels among non-allergic control subjects and

mild to moderate/severe asthmatics. In healthy subjects, mean serum IL-17 levels were below the detection threshold (Figure 1b). In accordance with FeNO levels, a significantly higher proportion of mild to severe persistent asthmatics had high levels of serum IL-17 values than mild intermittent asthmatics ($p < 0.01$). Mean serum IL-17 levels were high in moderate to severe persistent asthmatic subjects when compared to mild intermittent to persistent asthmatic subjects ($p < 0.01$), as summarized in Figure 1b.

Increased of CD4⁺ IL-17A⁺ T cells, serum IL-17, and FeNO correlated with asthma severity.

In this study, we have investigated serum IL-17 and FeNO expression levels in relation to the GINA-based asthma severity and control classification as assessed by C-ACT scores. Serum IL-17 levels were higher in the uncontrolled (mean $\pm$ SE, 43.06 ± 52.33 pg/ml) and partly controlled groups (12.52 ± 12.67 pg/ml) than in the controlled group (4.85 ± 5.46 pg/ml) ($p < 0.01$). The controlled group had lower FeNO levels than the partly controlled (20.03 ± 11.01 ppb vs. 28.93 ± 20.17 ppb, $p = 0.02$) and uncontrolled (20.03 ± 11.01 ppb vs. 54.72 ± 30.92 ppb, $p < 0.01$) groups. Plasma IL-17 levels were positively correlated with exhaled NO (Spearman's correlation $\rho = 0.74$; $p < 0.01$) and negatively correlated with C-ACT ($\rho = -0.63$; $p < 0.01$).

Pro-inflammatory Th17 cells, producing IL-17, have been identified in severe asthmatics and may contribute to airway inflammation in the pathophysiology of asthma. In support of this hypothesis, we found that the percentage of CD4⁺ IL-17A⁺ T cells was higher in the moderate to persistent asthmatic group than in the mild persistent asthmatic ($3.53 \pm 1.09\%$ vs. $1.56 \pm 0.60\%$, $p < 0.01$) and mild intermittent ($3.53 \pm 1.09\%$ vs. $1.08 \pm 0.60\%$, $p < 0.01$) groups (Figure 3b). As with CD4⁺ IL-17A⁺ T cells, we found percentage of CD4⁺ IL-4⁺ T cells was higher in the moderate to persistent asthmatic group than in the mild persistent asthmatic ($20.71 \pm 3.74\%$ vs. $14.59 \pm 5.48\%$, $p < 0.01$) and mild intermittent ($20.71 \pm 3.74\%$ vs. $10.06 \pm 5.36\%$, $p < 0.01$) groups (Figure 3c). CD4⁺ IL-4⁺ T cells levels were positively correlated with FeNO levels ($\rho = 0.69$; $p < 0.01$) (Figure 3d) and positively correlated with CD4⁺ IL-17A⁺ T cells levels ($\rho = 0.62$; $p < 0.01$) (Figure 3e).

IL-17 antagonist decreased iNOS expression in sputum cells from asthmatics.

To confirm IL-17 could be a potent activator of inducible nitric oxide synthase (iNOS) in allergic asthma, we collected sputum cells from asthmatics which treated with IL-17 antagonist. Moderate to severe asthmatics (n=5) exhibited greater iNOS production in sputum cells when compared to mild intermittent asthmatics (n=5) (Figure 4). Decreased iNOS expression in sputum cells was noted in all subjects after IL-17 neutralizing antibody ($p < 0.05$) (Figure 4).

Discussion

This study demonstrated that CD4⁺IL-17A⁺ cells, serum IL-17 levels, and FeNO levels from persistent asthmatic patients were higher than those from intermittent asthmatics or healthy controls, suggesting that airway and systemic inflammation occur in patients with persistent asthmatics. The significant positive correlation between serum IL-17 and FeNO levels, their negative correlation with C-ACT scores, and IL-17 antagonist could decrease iNOS expression by sputum cells from asthmatic subjects all suggested a cause-effect relationship in asthmatics. Results suggest higher CD4⁺IL-17A⁺ T cells counts from PBMCs play a key role in T cell-mediated airway inflammation with higher FeNO levels in persistent asthmatic children. The discovery of Th17 response and FeNO in airway inflammatory diseases has added an additional level of complexity for understanding the pathogenesis of persistent allergic asthma.

Recent evidence suggests that IL-17 plays a role in the sustained inflammation seen in allergic diseases (29, 30). Elevation of serum IL-17 levels up to 20 pg/mL have been considered to be an independent risk factor for severe asthma (31). In our study, the highest serum IL-17 levels were also found in the group of patients with severe persistent asthma (Figure 1 b). In accordance with this observation, higher serum IL-17 levels were detected in persistent asthmatics than in intermittent asthmatics, implying that the blood levels of this

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cytokine might be associated with the natural progression of severe asthma. As the severity of persistent asthma increased in the patients, the average FeNO levels also increased, which supported a role for IL-17 in maintaining airway inflammation. All the healthy children in this study had low to undetectable serum IL-17 levels, which suggested that the increased serum IL-17 levels could be a useful diagnostic aid for mild to severe persistent asthmatics.

Many severe asthma patients fail to respond satisfactorily to standard steroid therapy, and this observation has been linked to the presence of Th17 cells and neutrophilic inflammation in their airways (7, 8). Increased IL-17A expression has been observed in the bronchial submucosa of moderate to severe asthmatics, and neutrophilic inflammation may contribute to airway gland hypersecretion and airway wall remodelling in asthma (32-34). Furthermore, it was found that increased bronchial hyperreactivity in response to methacholine in patients with asthma was positively correlated with IL-17A levels in the sputum (35). Results from our study indicate that the percentage of CD4⁺IL-17A⁺ T cells showed a tendency to be higher in persistent asthmatics for the duration of the active disease, and the number of CD4⁺IL-17A⁺ T cells was higher in the moderate to severe persistent group than in the mild persistent and mild intermittent groups, as classified by using the GINA-based criteria.

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In our study, FeNO levels were more sensitive inflammation markers than the ordinary pulmonary functions, and revealed significantly higher in mild to severe persistent asthmatics than in mild intermittent asthmatics. In addition, there was a positive relationship between IL-17 production and disease severity. Several studies have established that the proinflammatory action of IL-17 depends considerably on its ability to induce inducible NOS (iNOS) and significantly stimulate NO production in various cell types (24-26). However, whether IL-17 directly contributes to iNOS expression in respiratory epithelial cells has yet been established. Increased inflammatory transcription factor nuclear factor- κ B (NF- κ B), which has been associated with airway inflammation in asthmatics, may explain the pathophysiological process by which IL-17 drives iNOS production (36, 37).

Identifying the different inflammatory phenotypes that exist in patients with asthma is crucial to develop better a therapeutic approach (38-40). Classification of severe asthma phenotypes by FeNO levels provides an independent classification of the severity of asthma (41). There are several possible explanations exist to account for the presence of high FeNO levels in patients with severe asthma. One of these may be the relative resistance of these patients to corticosteroid therapy. Measurements of FeNO levels in patients with chronic severe asthma showed that these patients, who are refractory to conventional treatments, might not respond to corticosteroids (41, 42). Patients with severe asthma and high FeNO

levels had a tendency to be younger, to exhibit the greatest airway reactivity, and to have the least awareness of their asthma symptoms (43, 44). The findings provide evidence that FeNO levels provide a valuable tool for classifying asthma phenotypes and allow the identification of a particularly worrisome subgroup of patients with persistent asthma (45).

In conclusion, our findings suggest that increased FeNO levels in the airways, in parallel with CD4⁺IL-17A⁺T cells from PBMCs and serum IL-17 levels, were found in patients with mild to severe persistent asthma, even in those who were treated with inhaled corticosteroids. In these patients, high systemic Th17 levels were negatively correlated with C-ACT scores, which suggested a substantial level of systemic inflammation. Persistent asthma is a complex chronic allergic airway inflammatory disease involved in Th17 mediated FeNO production. Clarifying the pathway linked to asthma severity may benefit development of novel therapeutics to prevention of severe asthma.

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Figure legends

Figure 1. (a) Mean FeNO levels and (b) serum IL-17 levels in GINA guideline-based asthma severity measure groups. (* mean $p < 0.05$)

Figure 2. Associations between (a) serum IL-17 and FeNO, (b) serum IL-17 and C-ACT scores, and (c) serum IL-17 and inhaled corticosteroid usage (eq Fluticasone/day) in asthmatic children. r : Spearman's rho correlation coefficient.

Figure 3. Intracellular IL-17A and IL-4 in CD4⁺ T cells in asthmatic children. PBMCs were stimulated with anti-CD3 mAb (2 µg/ml) for 5 days, followed by stimulation with PMA (10 ng/ml) plus ionomycin (1 µg/ml) for the last 5 hours, and addition of brefeldin A (10 µg/ml) for the final hour. (a) Intracellular expression of IL-17A and IL-4 was measured by gating CD4⁺ T cells using flow cytometry. Representative figures are shown. (b) The IL-17A+CD4⁺ T cells and (c) IL-4+CD4⁺ T cells (%) of experiments for mild intermittent (n=20), mild persistent (n=20), and moderate-to-severe persistent subjects are shown (n=10) (* indicates $p < 0.05$). The relationship between IL-4+CD4⁺ T cells (%) versus (d) FeNO and (e) IL-17A+CD4⁺ T cells (%) in asthmatic children. r : Spearman's rho correlation coefficient.

Figure 4. iNOS expression in sputum cells from asthmatic children. (a) Sputum cells (2×10^6 cells/mL) were treated with IL-17 neutralizing antibody (40ng/mL) for 1 day from mild intermittent asthmatics (n=5) and moderate-to-severe asthmatics (n=5). iNOS expression was determined by immunoblot analysis. (b) Statistical data of experiments on paired samples (* $p < 0.05$; between mild intermittent and moderate-to-severe persistent asthmatics). (# $p < 0.05$ as compared with IL-17 neutralizing antibody).

Table 1. Patient characteristics and pulmonary function in asthmatic patients with different severity

GINA severity	Mild intermittent	Mild persistent	Moderate to Severe persistent
Health subjects			
Number	42	42	36
20			
Mean age (yr)	7.74±2.24 ⁺		9.66±3.44* ⁺
10.67±3.16* ⁺	13.06±3.49		
Gender (M:F)	26:16	29 : 13	22 :
14	12 : 8		
Total IgE (IU)	393.7±359.1		793.2±656.2*
1345.3±1099.3* [#]			
Der p (class)	3.01±2.04		4.69±1.70*
5.03±1.12*			
Neutrophils (number/μl,%)	58.90±18.45		58.68±19.45
61.46±21.84			
Eosinophils (number/μl,%)	1.74±2.18		2.49±1.89
2.95±1.92			

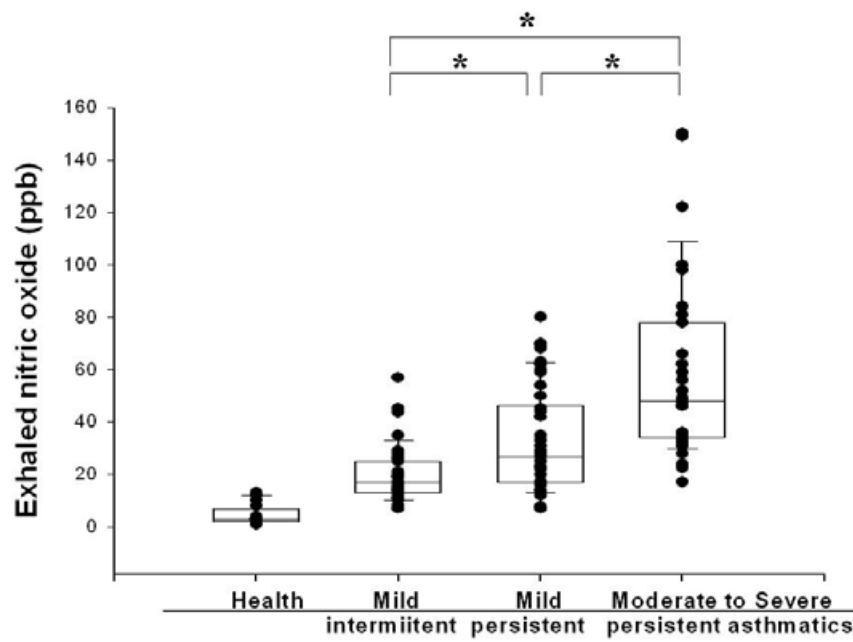
C-ACT	25.90±2.52	24.39±2.08*
19.47±3.46* [#]		
FEV1 (% predicted)	98.53±11.62	93.21±15.19
69.99±17.64* [#]		
FEV1/FVC (%)	99.70±7.34	95.22±9.42
75.44±16.93* [#]		
MMEF (%)	77.34±22.34	76.49±23.76
55.68±16.01* [#]		
PEFR (%)	105.59±40.32	89.89±18.54
71.59±16.50* [#]		
Inhaled CS	--	109.5 ±29.4
220.0±123.2 [#]		
(eq fluticasone/day)		

Data are presented as mean ± SE; * $p < 0.05$ vs. mild intermittent asthma; [#] $p < 0.05$ vs. mild persistent asthma; ⁺ $p < 0.05$ vs. health subjects

FeNO=Fractional exhaled nitric oxide; FEV1= Forced expiratory volume in the first second; FVC= Forced vital capacity;

MMEF= Maximal mid-expiratory flow; PEFR= Peak expiratory flow; CS=Corticosteroids, U.D.= under-detectable

(a)



(b)

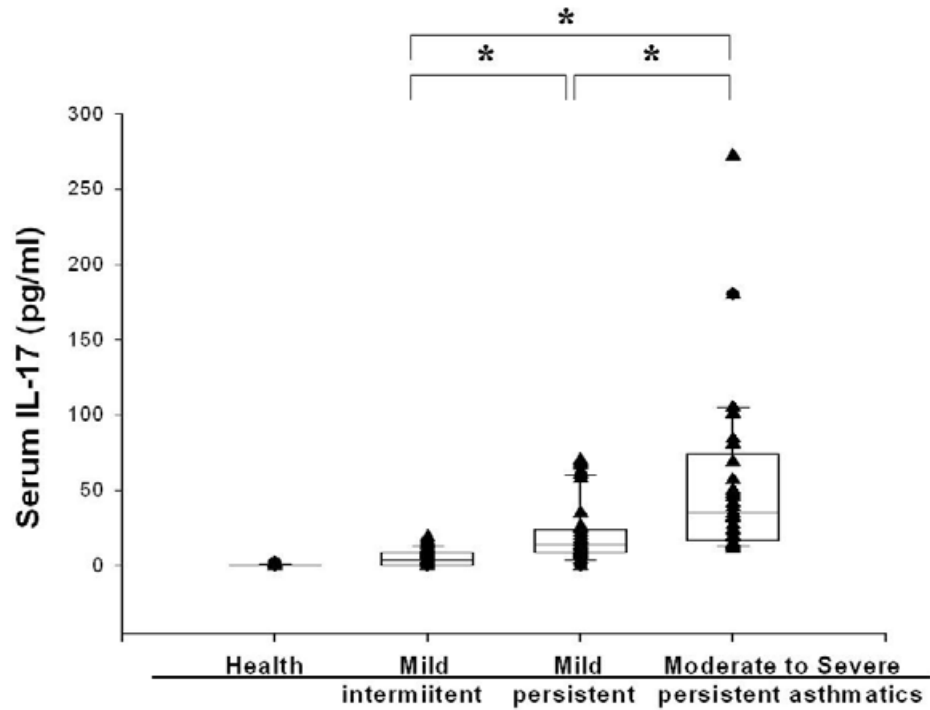
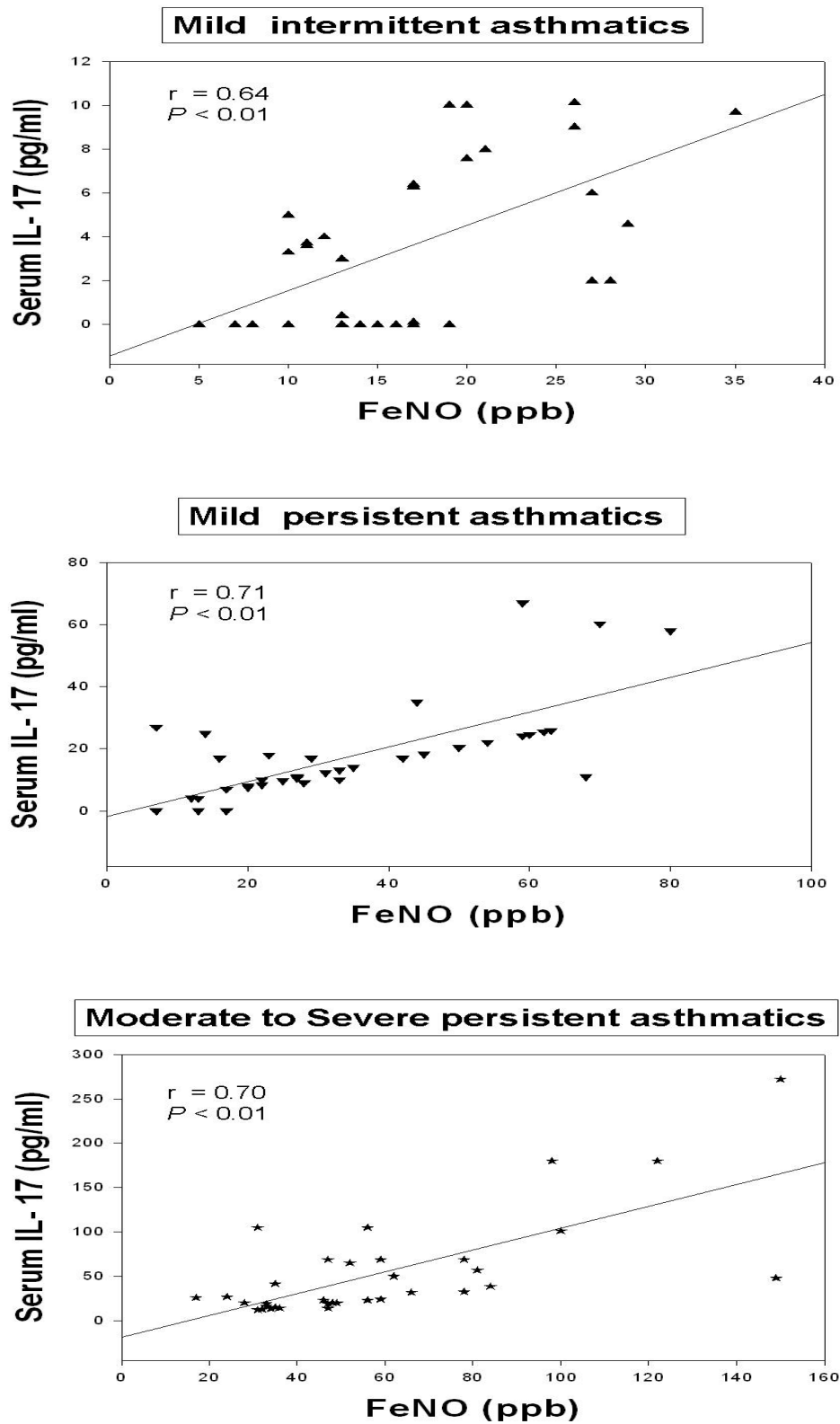
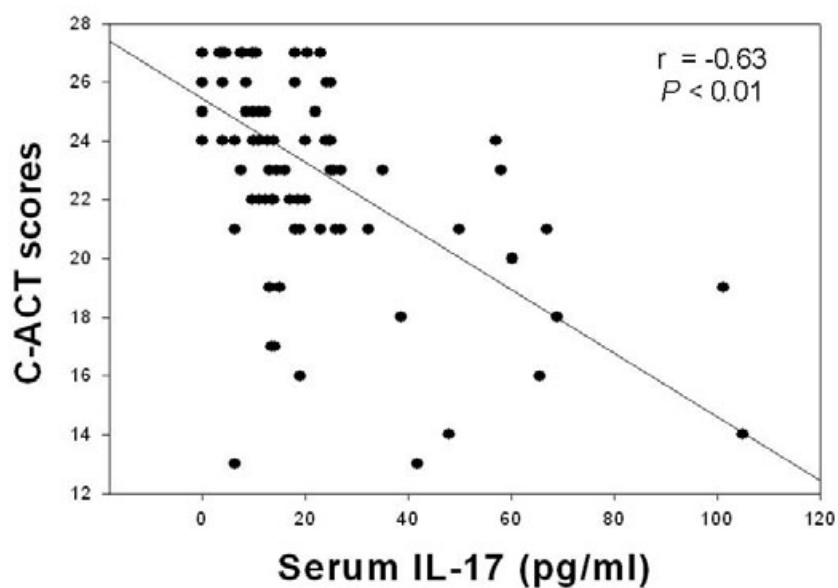


Figure 1

(a)



(b)



(c)

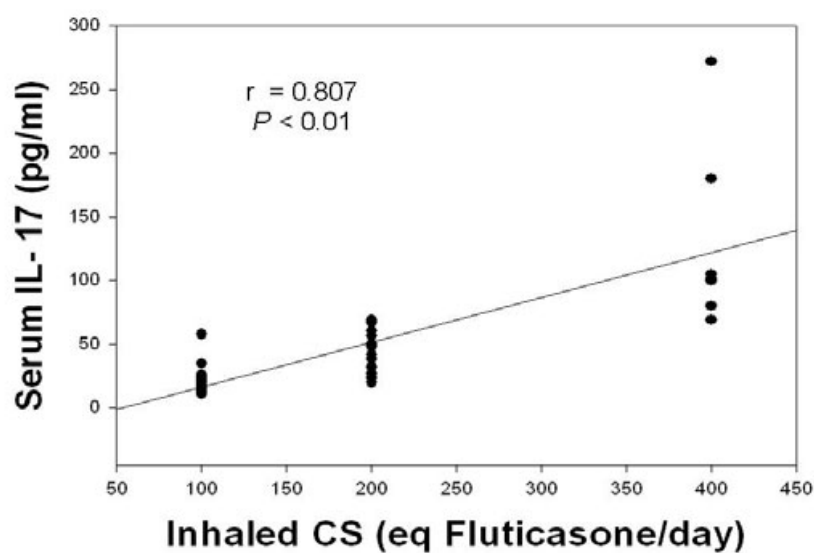
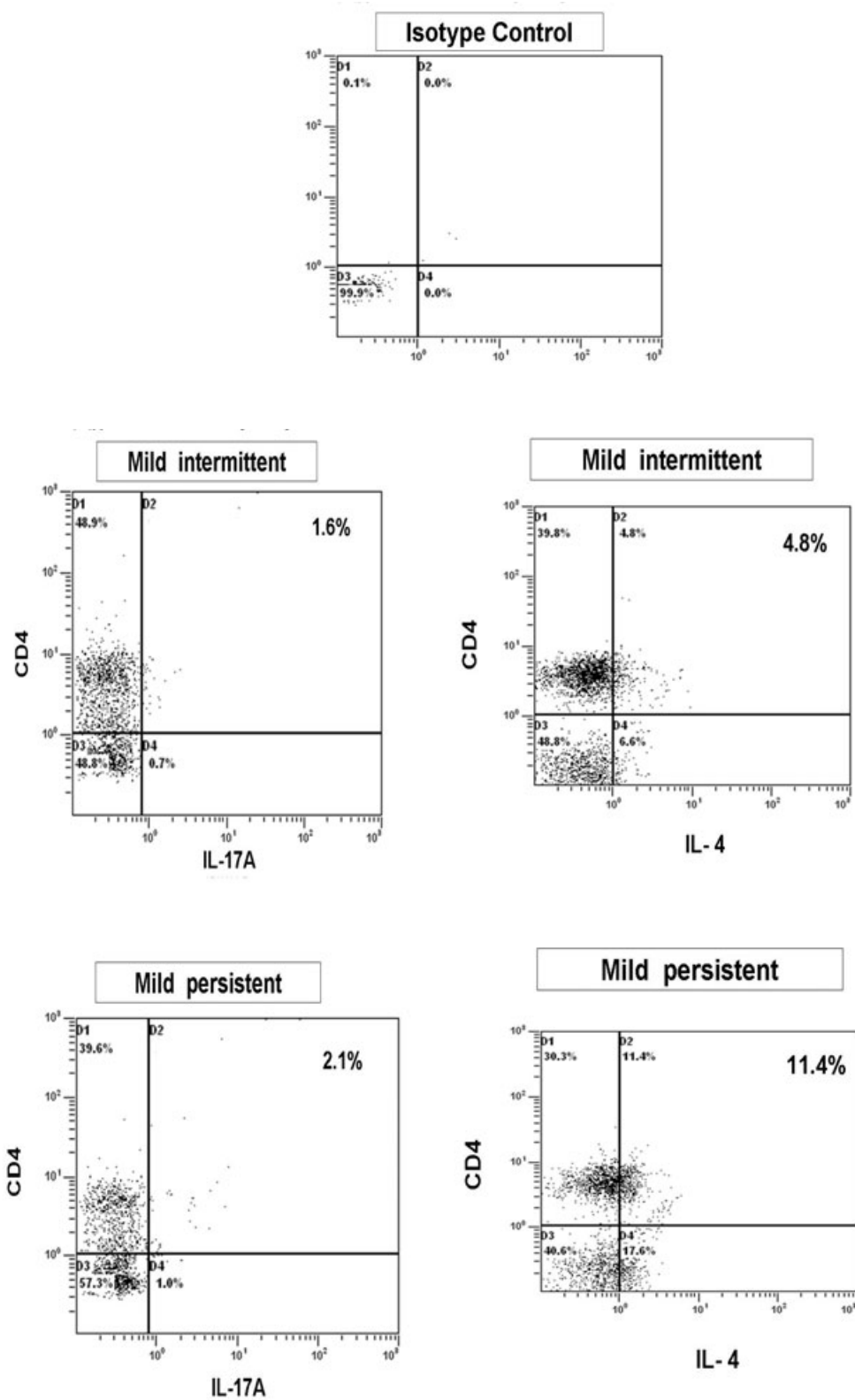
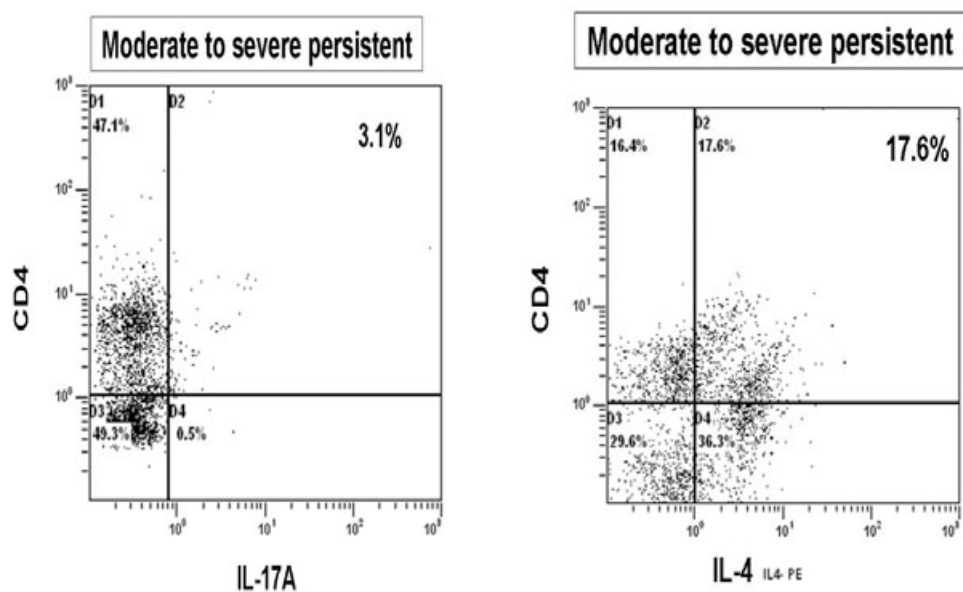


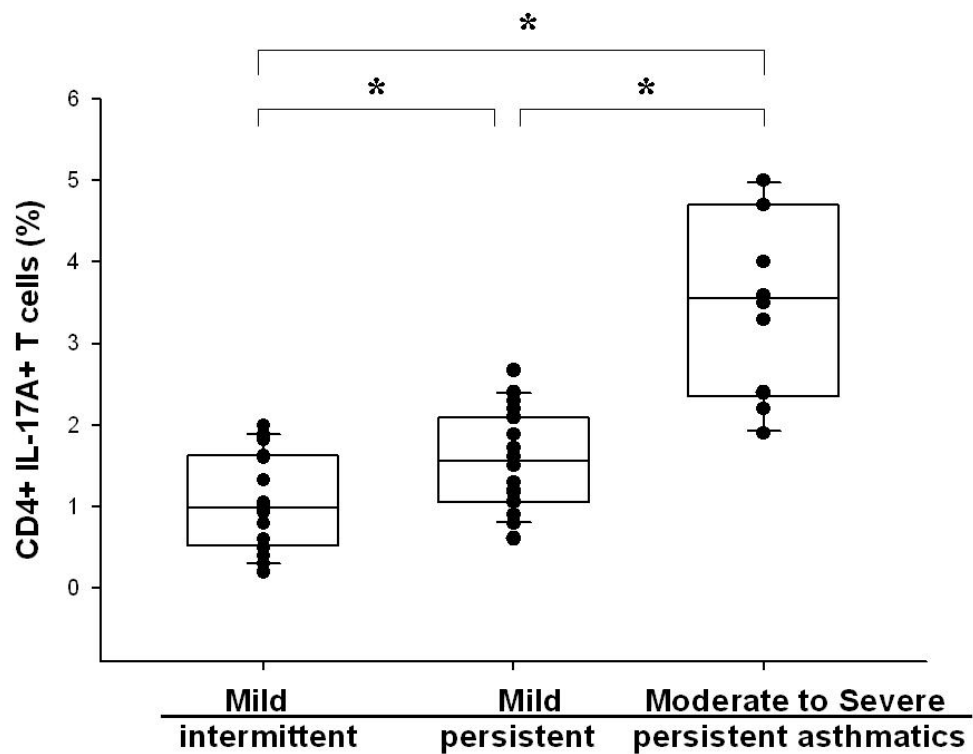
Figure 2

(a)

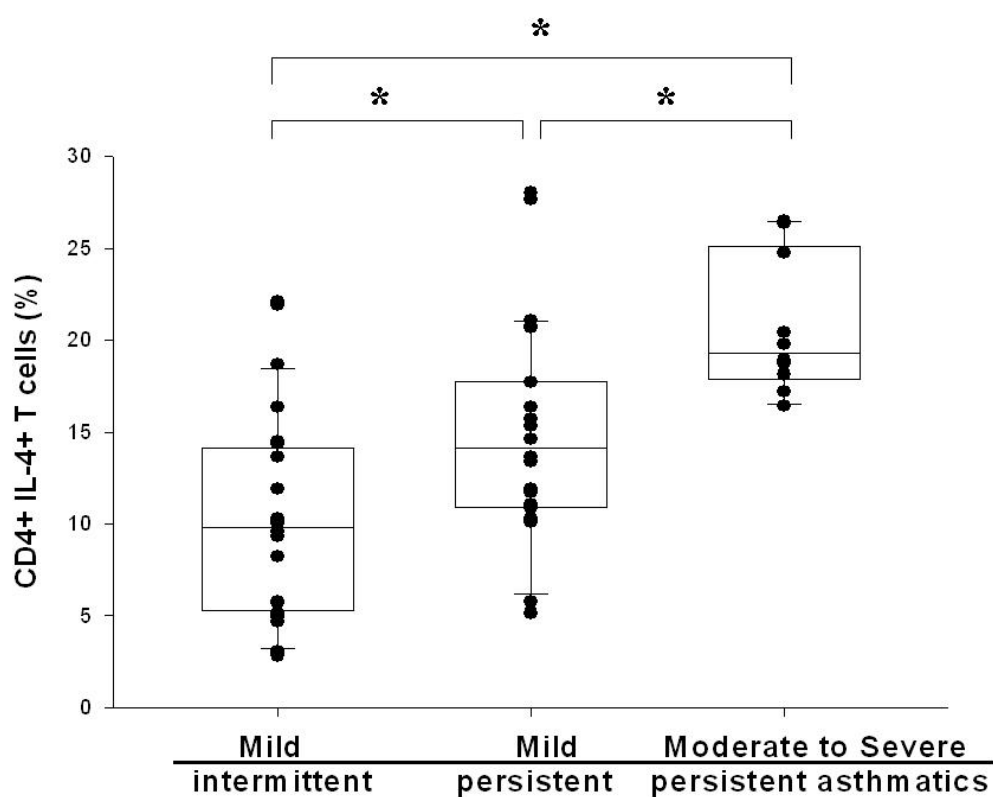




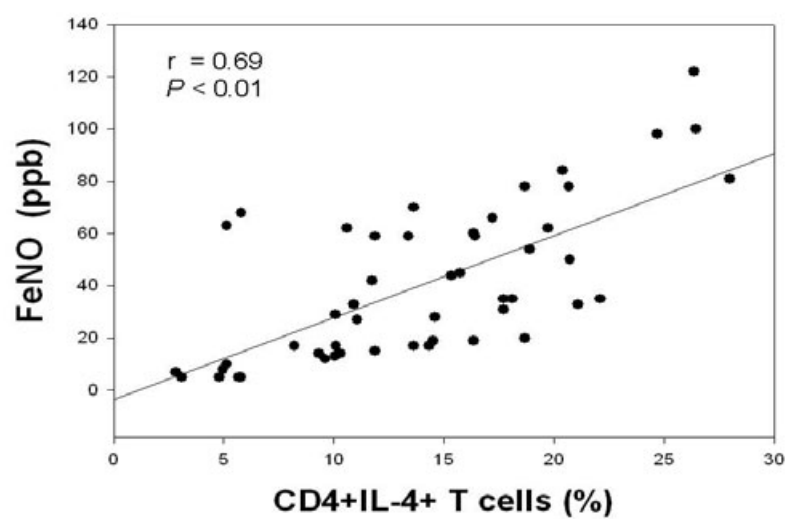
(b)



(c)



(d)



(e)

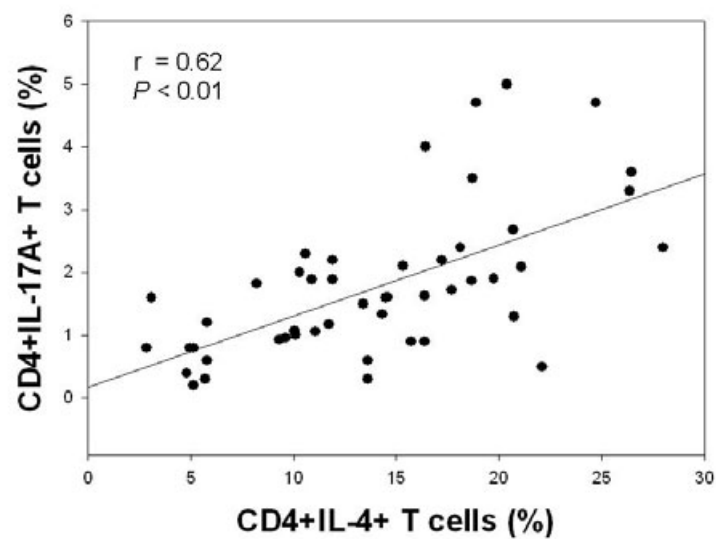
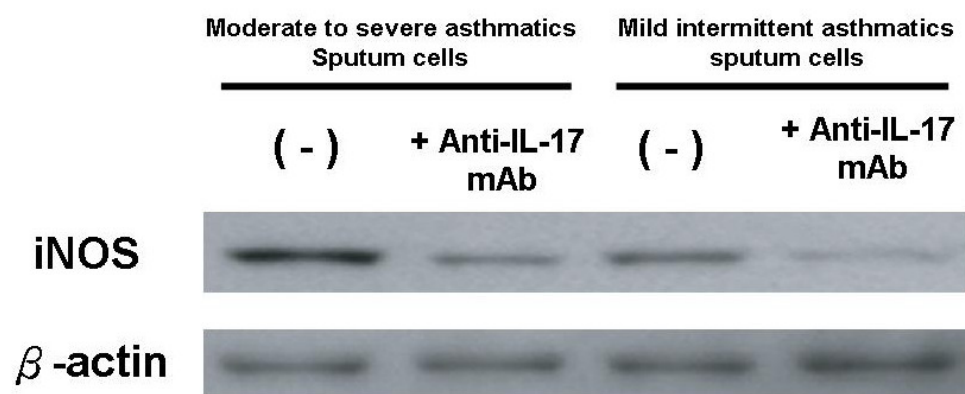


Figure 3

(a)



(b)

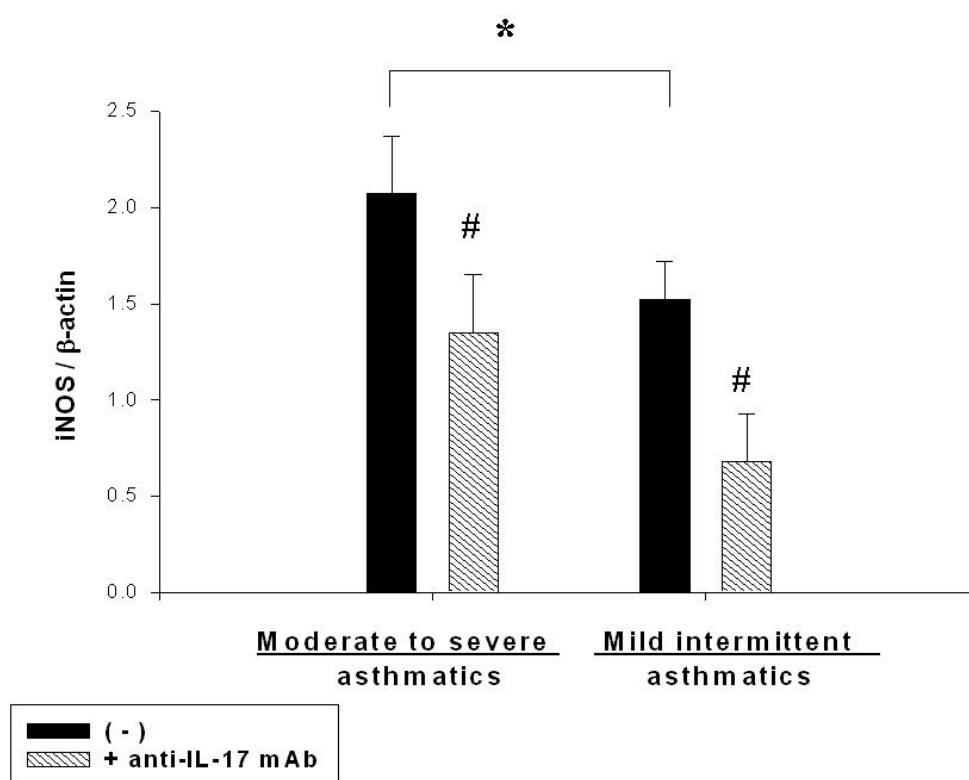


Figure 4