

Aberrant Expression of Regulatory Cytokine IL-35 and Pattern Recognition Receptor NOD2 in Patients with Allergic Asthma

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Abstract—We investigated the plasma concentration of the novel regulatory cytokine IL-35 and intracytosolic pattern recognition receptors nucleotide-binding oligomerization domain (NOD)-like receptors in granulocytes and explored their potential implication in disease severity monitoring of allergic asthma. The expression of circulating IL-35 and other pro-inflammatory mediators in asthmatic patients or control subjects were evaluated using enzyme-linked immunosorbent assay (ELISA). The intracellular expressions of NOD1 and NOD2 in CCR3+ granulocytes were assessed using flow cytometry. Plasma concentrations of IL-35, IL-17A, basophil activation marker basogranulin, and eosinophilic airway inflammation biomarker periostin were significantly elevated in allergic asthmatic patients compared to non-atopic control subjects (all probability (p) <0.05). Both granulocyte markers exhibited significant and positive correlation with plasma IL-35 concentration in asthmatic patients (all p <0.05). Significant positive correlation was also identified between plasma concentrations of IL-35 and periostin with disease severity score in asthmatic patients (both p <0.05). The basophil activation allergenicity test was positive in allergic asthmatic patients but not in control subjects. Despite significantly elevated eosinophil count in allergic asthmatic patients, downregulation of NOD2 in CCR3+ granulocytes was observed in these patients (both p <0.05). A negative correlation between plasma concentrations of tumor necrosis factor family member LIGHT and soluble herpesvirus entry mediator was observed in patients with elevated plasma concentration of IL-35 (p <0.05). Aberrant expression of NOD2 in granulocytes may be contributed to the impaired innate immunity predisposing allergic asthma. IL-35 may serve as a potential surrogate biomarker for disease severity of allergic asthma.

KEY WORDS: asthma; basogranulin; granulocytes; IL-35; NOD2; periostin.

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INTRODUCTION

The prevalence and incidence of atopic diseases including atopic dermatitis, allergic rhinitis, and allergic asthma have been increasing in developed countries over the past several decades [1]. Allergic asthma is a complex and heterogeneous disease characterized by spontaneous airflow limitation, lung tissue remodeling, increased plasma IgE concentration, and airway hyperresponsiveness, with activation of the infiltration of T lymphocytes, type 2 innate lymphoid cells, granulocyte eosinophils, and basophils into the airway submucosa [2–4]. Activation and

degranulation of eosinophils and basophils play critical roles in the inflammatory process of allergic asthma [4].

Eosinophils are the most important inflammatory effector cells infiltrating and accumulating at the site of allergic inflammation, *e.g.*, the airway submucosa, through the mediation of adhesion molecules [5]. Activated eosinophils release cytotoxic and inflammatory molecules such as major basic protein, eosinophil peroxidase, eosinophilic cationic protein, superoxides, cysteinyl leukotrienes, and cytokines and chemokines [5]. These molecules orchestrate epithelial and epidermal damage, bronchoconstriction and mucus secretion, eventually resulting in the manifestation of allergic asthma [5, 6]. Apart from eosinophils, our previous study has shown that the T helper 17 (Th17) cytokine IL-17A cytokine can induce the release of inflammatory cytokines and chemokines upon the interaction of human basophils with bronchial epithelial cells, implicating an important pathological role of basophils in allergic asthma [7].

Dysregulation of cytokines has been shown to relate with the immunopathogenesis of allergic asthma [8–10]. IL-35 has been identified as a novel immunosuppressive/anti-inflammatory regulatory cytokine. It is a dimeric protein with two subunits, IL-12A (p35) and Epstein-Barr virus-induced 3 (EBI3) [11, 12]. IL-35 is the newest member of the IL-12 family including IL-23 and IL-27. Within the CD4⁺ helper T (Th) cell population, IL-35 is expressed by resting and activated regulatory T cells (Tregs/Tr35) by converting conventional T cells (Tconv) into IL-35-dependent-induced Tregs (iTTr35) [12, 13], but not effector T (Teff) cells [12]. iTTr35 cells are strongly suppressive and stable without the expression of Treg transcription factor forkhead box P3 (Foxp3) [13]. Maximal expression and secretion of IL-35 by Tregs require their contact with Teff cells, which potentiates the suppressive capacity of Tregs [11]. Similar to that of regulatory cytokine transforming growth factor (TGF)- β and IL-10, IL-35 is the major component of the suppressive repertoire [13]. IL-35 directly suppresses *in vitro* Teff cell proliferation in the absence of IL-10 or TGF- β [14]. The gp130 and IL-12R β 2 chain together constitute the receptor of IL-35 [15]. IL-35 can signal through the homodimerization of either IL-12R β 2 or gp130, as well as IL-12R β 2:gp130 heterodimerization [15]. However, the combined IL-12R β 2:gp130 receptor is required for maximal suppression, IL-35 induction, and iTTr35 conversion. A previous animal study has shown that IL-35 production by inducible costimulator-positive Treg cells can suppress inflammatory IL-17 production and subsequently reverse established IL-17-dependent airway hyperresponsiveness [16].

Periostin/osteoblast-specific factor-2 is a systemic biomarker of airway eosinophilia in asthmatic patients with potential therapeutics utility in asthmatic patients with Th2 inflammation [17]. It is a secreted matricellular protein associated with fibrosis, and its expression is up-regulated by Th2 cytokine IL-4 and IL-13 in bronchial epithelial cells [18, 19] and bronchial fibroblasts [20]. Elevated expression of periostin has been found in asthmatic mice [21] and bronchial epithelial cells of asthmatic patients [18]. In asthmatic patients, periostin expression levels correlate with reticular basement membrane thickness, an indicator of subepithelial fibrosis [22].

Moderate pulmonary bacterial infection, such as that by *Chlamydia pneumoniae*, can provoke allergic inflammation upon the activation of innate immunity-related dendritic cells by inhaled allergen [23–25]. The innate immune system recognizes microorganisms through pattern recognition receptors (PRRs), including toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and RIG-I-like receptors (RLRs) which detect conserved microbial components called pathogen-associated molecular patterns (PAMPs). The most widely expressed and well-characterized TLRs on the cell surface and in endosomes recognize microbes, whereas NLRs and RLRs detect bacterial and viral components in the cytosol [26]. Among these PRRs, members of the intracellular NLRs family including NOD1 and NOD2 have been identified as key mediators in inflammatory and immune responses, by recognizing intracellular microbes and commensal organisms [27–29]. NOD2 can also function as a cytoplasmic viral PRR by producing of interferon (IFN)- β [30]. Regarding antibacterial innate immunity, NOD1 and NOD2 can sense the cytosolic presence of the peptidoglycan fragment γ -D-glutamyl-mesodiaminopimelic acid (iE-DAP) in all Gram-negative and certain Gram-positive bacteria, and muramyl dipeptide (MDP) in almost all bacteria, respectively [26, 27, 31]. Bacterial sensor NOD2 can trigger a potent antigen-specific immune response with a Th2-type polarization cytokine profile [32]. The crucial pathological roles of NOD1 and NOD2 in immune disorders have been proven from the linkage of NOD1 and NOD2 polymorphisms with chronic inflammatory Crohn's disease, atopic dermatitis, atopic eczema, and allergic asthma [33–35]. We and other group have reported NOD1 and NOD2-mediated activation of eosinophils and basophils in allergic inflammation [36–38]. To further elucidate immunopathological mechanisms mediating allergic inflammation in allergic asthma, we investigated the expression of plasma IL-35 and NOD in CCR3⁺ granulocytes including eosinophil, as well as

their relationships with disease activity and relevant granulocyte activation markers in patients with allergic asthma.

PATIENTS AND METHODS

Asthmatic Patients, Non-allergic Control Subjects and Blood Samples

Twenty-four Chinese patients aged 13 to 30 years with asthma were recruited from Allergy Clinic of our university-affiliated teaching hospital. The diagnosis of asthma was made according to the American Thoracic Society criteria [39]. Lung function of the subjects was assessed by prebronchodilator spirometry (Model S, Vitalograph, Buckingham, UK) according to the American Thoracic Society standards [40]. The results were compared with the local predicted age- and sex-matched values [41]. The disease severity was presented as disease severity score (DSS) (6–30 points) that assessed exercise tolerance, the frequency of acute asthma exacerbation, the use of antiasthma medications during the 6 months before evaluation, and the occurrence of cough, wheezing, or chest tightness in the daytime and at night [42]. Antiasthma treatments in our patients were stable for at least 4 weeks before study. Twenty-four age and sex-matched non-allergic control subjects were recruited. They did not have any history of allergic or inflammatory disease and did not require any drug treatment at the time of study. All subjects were free from respiratory tract infection, asthma exacerbation, and the use of systemic corticosteroids for 4 weeks prior to study. The atopic status of these patients and control subjects was ascertained by positive serum-specific IgE assays to house dust mite *Dermatophagoides pteronyssinus* (*D. pteronyssinus*), cat dander, dog dander, mixed cockroaches, and mixed molds by fluorescence enzyme immunoassay (AutoCAP analyzer, Pharmacia Diagnostics AB, Uppsala, Sweden) [43]. Sixteen milliliters (mL) of EDTA-anticoagulated and 3 mL clotted venous peripheral blood were collected from each patient and control subject. Plasma and serum samples were preserved at -80°C for subsequent assays. All the enrolled subjects were ethnic Chinese. The above protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong-New Territories East Cluster Hospitals, and informed written consent was obtained from all subjects or their parents in

accordance with the 1964 Declaration of Helsinki and its later amendments.

Flow Cytometric Analysis for NOD1 and NOD2 Expression in CCR3+ Granulocytes from Asthmatic Patients and Controls

Peripheral blood granulocytes from asthmatic patients and control subjects were purified by Percoll gradient centrifugation (GE Healthcare Life Sciences, NJ, USA). The expression of NOD2 in granulocytes of asthmatic patients and control subjects was determined by flow cytometry (BD FACSCalibur, BD Biosciences Corp., CA, USA) as previously described [38, 44]. Murine serum-blocked granulocytes were fixed and permeabilized using Fix/Perm solution (BD Biosciences, Mississauga, ON, Canada) for the intracellular staining of NOD1 and NOD2. Unconjugated mouse anti-NOD1 (Abcam Inc., MA, USA), anti-NOD2 antibody (BioLegend Corp., CA, USA), corresponding mouse IgG1, κ isotypic control antibody (BD Pharmingen Corp., San Diego, CA, USA), together with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H + L) secondary antibodies (Zymed Laboratories, Inc., CA, USA), were used for intracellular staining. The expression of NOD1 and NOD2 in final granulocyte suspension with 10,000 events was assessed using flow cytometry with gating by CCR3, a specific chemokine receptor on eosinophils and basophils. Results were expressed as mean fluorescence intensity (MFI).

Quantitative Analysis of Cytokines, Periostin and Basogranulin

Plasma IL-35, IL-17A, periostin and basogranulin concentrations were measured using enzyme-linked immunosorbent assay (ELISA) reagents from BioLegend and high sensitive ELISA reagent from eBioscience Inc., CA, USA, ELISA reagent from Raybiotec Inc., GA, USA and ELISA reagent from Cusabio Biotech Co., Ltd., China, respectively. Serum concentration of airway inflammatory cytokine LIGHT (lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator (HVEM) on T cells/CD258) and plasma concentration of LIGHT receptor soluble HVEM were measured using ELISA kits from R&D Systems, MN, USA and Abcam Inc., MA, USA, respectively.

Assay of Serum IgE

Serum total IgE and specific IgE against house dust mite (Der p1), cat and dog dander, mixed cockroaches, and mixed molds were measured to ascertain the atopic status

of all participants using fluorescence enzyme immunoassay (ImmunoCAP immunoassay analyzer, Phadia, Uppsala, Sweden) [9, 44].

Basophil Activation Test (BAT)

To further assess the IgE-mediated allergic inflammation, we evaluated the functional basophil-bound-specific IgE using specific allergen-mediated BAT (Allergen Assay Kit, Beckman Coulter Corp., FL, USA). This is a cellular functional analysis based on flow cytometric analysis of the IgE-mediated up-regulation of the cell surface expression of activation marker CD203c associated with cellular degranulation on basophils upon the *ex vivo* allergen challenge such as house dust mite, cockroaches, and cat dander [45]. Using an accurate basophil gating tool (CRTH2+ CD203c+ CD3-), BAT was performed on whole blood specimen by flow cytometer (Navios Flow Cytometer, Beckman Coulter).

Statistical Analysis

The Mann-Whitney rank sum test was used to compare the differences between different groups, and Spearman's rank correlation test was used to assess the correlations among different measurable parameters. All analyses were performed using Statistical Package for the Social Sciences (SPSS) statistical software for Windows, Version 20 (SPSS Inc., IL, USA). A probability (p) <0.05 was considered as statistically significant.

RESULTS

Asthmatic Patients and Control Subjects

The clinical characteristics of the study population are summarized in Table 1. Twelve non-steroid-treated asthmatic patients, 12 steroid-treated asthmatic patients, and 24 control subjects were enrolled in this study, with their mean±SD ages

Table 1. Demographic, Clinical, and Laboratory Data of the Allergic Asthmatic Patients With or Without Receiving Inhaled Corticosteroid and Control Subjects

Parameters	Non-steroid-treated patients (n=12)	Steroid-treated patients (n=12)	Control subjects (n=24)
Sex (male/female)	3/9	5/7	12/12
Age at evaluation, year (mean±SD, range)	20.8±5.7 (13–29)	22.1±4.5 (16–30)	23.9±4.7 (13–32)
Spirometry			
FEV1, L	2.8 (2.6–3.2)	2.9 (2.5–3.5)	N.A.
FEV1, (% predicted)	84 (79.0–92.5)	80.5 (74.0–99.5)	N.A.
Disease severity score (DSS)	9 (8–9)	14 (11–16) ####	N.A.
Eosinophil % in peripheral blood	5.0 (3.5–6.0)****	5.5 (5.0–7.0)****	2.0 (1.0–2.0)
Basophil % in peripheral blood	1.0 (0.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)
Serum total IgE (kIU/L)	533 (441–714)****	818 (417–2109)****	20 (12–37)
Presence of at least one			
Positive allergen-specific IgE, n (%)	12 (100)	12 (100)	0 (0)
<i>D. pteronyssinus</i> , n (%)	12 (100)	12 (100)	0 (0)
Cockroaches, n (%)	4 (33.3)	4 (33.3)	0 (0)
Cat dander, n (%)	2 (16.7)	4 (33.3)	0 (0)
Dog dander, n (%)	0 (0)	1 (8.3)	0 (0)
Mixed molds, n (%)	1 (8.3)	3 (25.0)	0 (0)
Positive basophil activation test			
House dust mite, <i>D. pteronyssinus</i> , n (%)	11 (91.6)	9 (75.0)	0 (0)
Cockroaches, Bla g2 protein, n (%)	2 (16.7)	2 (16.7)	0 (0)
Cat dander, Fel d1 protein, n (%)	12 (100)	10 (83.3)	0 (0)
Treatment with budesonide			
No. of patients (%)	N.A.	9 (75)	N.A.
Daily dose (µg)	N.A.	400±0	N.A.
Fluticasone propionate			
No. of patients (%)	N.A.	3 (25)	N.A.
Daily dose (µg)	N.A.	386.1±278.7	N.A.

Values were given as median (IQR) or mean±SD

**** p <0.0001 comparing with controls; #### p <0.0001 comparing between steroid and non-steroid-treated groups

at evaluation being 20.8 ± 5.7 , 22.1 ± 4.5 , and 23.9 ± 4.7 years, respectively ($p=0.101$). The FEV₁ (median (IQR)) of all asthmatic patients was 2.8 (2.6–4.5)L with 82.0 (77.0–94.5)% of predicted normal values. The DSS was found to be significantly higher in steroid-treated patients than that in non-steroid-treated patients 14 (11–16) vs 9 (8–9), $p<0.0001$. Eosinophils (%) but not basophils (%) and serum total IgE concentration were found to be significantly higher in asthmatic patients with or without steroid treatment compared to control subjects (all $p<0.0001$). All asthmatic patients were found to have specific IgE against allergen *D. pteronyssinus*, and some of them also have specific IgE against at least one of the following allergens including mixed cockroaches, cat dander, dog dander, and mixed molds. None of the control subjects presented with specific IgE against the above allergens. BAT results showed that none of the control subjects exhibited positive BAT, but positive BAT upon the challenge by house dust mite Der p1, cockroach Bla g2, and cat dander Fel d1 protein was found in 83, 16.7, and 91.6 %, respectively, of total asthmatic patients. Among all 24 recruited asthmatic patients, 12 (50 %) received inhaled corticosteroid treatment, including nine with budesonide (daily dose: 400 µg) and three with fluticasone propionate (daily dose: 386.1 ± 278.7 µg).

Plasma/Serum Concentrations of Regulatory Cytokine IL-35, IL-17A, Basogranulin, Periostin, and LIGHT

There were detectable IL-35, IL-17A, basogranulin, periostin, and LIGHT present in the plasma/serum of both control subjects and asthmatic patients. Plasma IL-35, IL-17A, basogranulin, and periostin concentrations were significantly higher in all allergic asthmatic patients (steroid and non-steroid treated) than in control subjects [median (IQR) of IL-35, IL-17A, basogranulin, and periostin for asthmatic patients vs controls: 55.9 (6.6–419.0) vs 2.5 (0.1–16.1)ng/mL, 2.8 (2.3–4.5) vs 2.2 (1.8–2.4)pg/mL, 712.5 (224.0–1181.0) vs 228.0 (131.0–348.0)pg/mL, and 107.0 (14.0–632.5) vs 1.0 (1.0–17.5)ng/mL, respectively, all $p<0.01$]. As illustrated in Fig. 1a–d, further analysis showed that plasma IL-35 and basogranulin concentrations were significantly higher in steroid-treated allergic asthmatic patients than those in controls [IL-35 and basogranulin for steroid-treated asthmatic patients vs controls: 32.7 (8.3–379.5) vs 2.5 (0.1–16.1)ng/mL and 618.5 (424.5–1172.0) vs 228.0 (131.0–348.0)pg/mL, respectively, all $p<0.005$] while plasma periostin and IL-17A concentrations were significantly higher in both non-steroid-treated and steroid-treated allergic asthmatic patients than that in controls [periostin and IL-17A for steroid and non-steroid-treated patients

vs controls: periostin, 187.0 (48.0–632.5) vs 1.0 (1.0–17.5) and 107.0 (6.0–632.0) vs 1.0 (1.0–17.5)ng/mL; IL-17A, 2.8 (2.2–4.9) vs 2.2 (1.8–2.4) and 2.9 (2.3–4.3) vs 2.2 (1.8–2.4)pg/mL, respectively, all $p<0.05$]. However, there was no significant difference of serum concentration of LIGHT between allergic asthmatic patients with or without steroid treatment and controls (both $p>0.05$).

NOD2 in CCR3+ Granulocytes

In order to assess bacterial-infection related innate immunity in allergic asthma, we investigated the expression of PRR NOD1 and NOD2 in CCR3+ granulocytes. As shown in Fig. 2a, b, the gated CCR3+ granulocytes could denote the eosinophil population in total granulocytes, which was confirmed by the strong correlation between the percentage of CCR3+ cells in granulocytes and the percentage of eosinophils in white blood cells ($r=0.86$, $p<0.001$). We did not observe any detectable protein expression of NOD1 in CCR3+ granulocytes (data not shown). Intracytosolic NOD2 expression was significantly lower in all allergic asthmatic patients (steroid and non-steroid treated) than in control subjects [median (IQR) of NOD2 (MFI) for asthmatic patients vs controls: 1.21 (1.06–1.54) vs 2.31 (1.41–2.84), $p=0.0006$]. As shown in Fig. 2c, d, further analysis found that NOD2 expressions (MFI) were significantly lower in allergic asthmatic patients with or without steroid treatment than those in controls [steroid-treated asthmatic patients vs controls: 1.17 (1.03–1.56) vs 2.31 (1.41–2.84) and non-steroid-treated asthmatic patients vs controls: 1.21 (1.08–1.48) vs 2.31 (1.41–2.84), both $p<0.01$].

Correlation Between Different Immunological Parameters and DSS in Asthmatic Patients

As shown in Table 2, only plasma concentrations of periostin showed significant and positive correlation with DSS in all asthmatic patients (steroid and non-steroid treated, $p<0.05$). Moreover, the plasma concentrations of IL-35 and periostin concentrations exhibited significant and positive correlation with DSS in steroid-treated asthmatic patients (Table 2). Further analysis showed that plasma IL-35 and periostin concentrations exhibited significant and positive correlation ($r=0.843$, $p=0.001$) in these patients (Fig. 3a). IL-35 concentration was also positively correlated with basogranulin concentration in all allergic patients (without or with steroid treatment, Fig. 3b, c, $r=0.859$, $p=0.001$; $r=0.673$, $p=0.020$, respectively). Plasma IL-35 concentration was negatively correlated with total IgE concentration in non-steroid-treated asthmatic patients

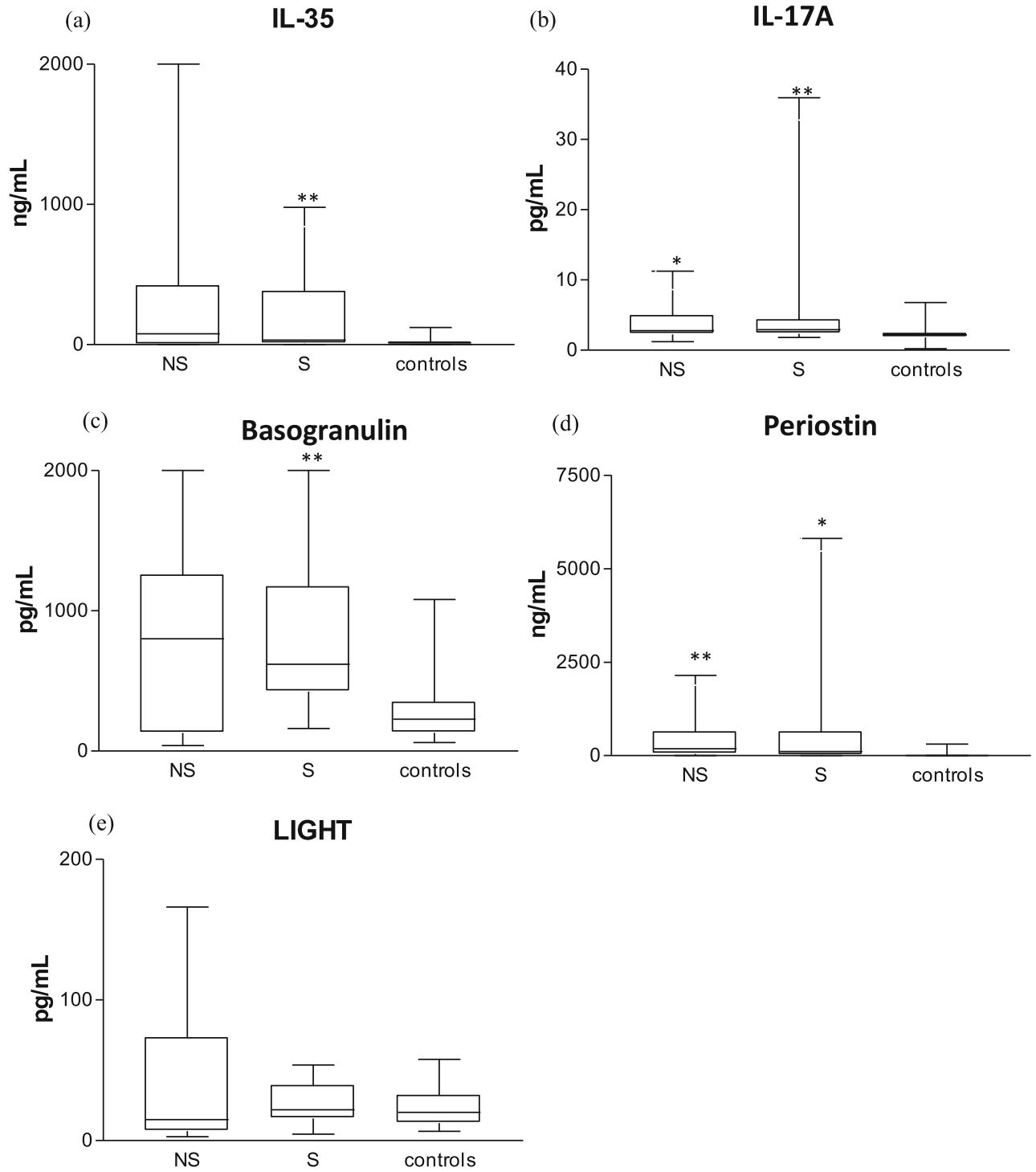


Fig. 1. Concentrations of IL-35, IL-17A, basogranulin, and periostin in plasma and LIGHT in serum. *Box* and *whisker* plots showing comparisons of plasma concentrations of **a** IL-35, **b** IL-17A, **c** basogranulin and **d** periostin, and **e** serum concentration of LIGHT between asthmatic patients with or without steroid treatment, and control subjects were determined by Mann-Whitney rank sum test. * $p < 0.01$, ** $p < 0.005$. *S* steroid-treated asthmatic patients, *NS* non-steroid-treated asthmatic patients, *controls* control subjects.

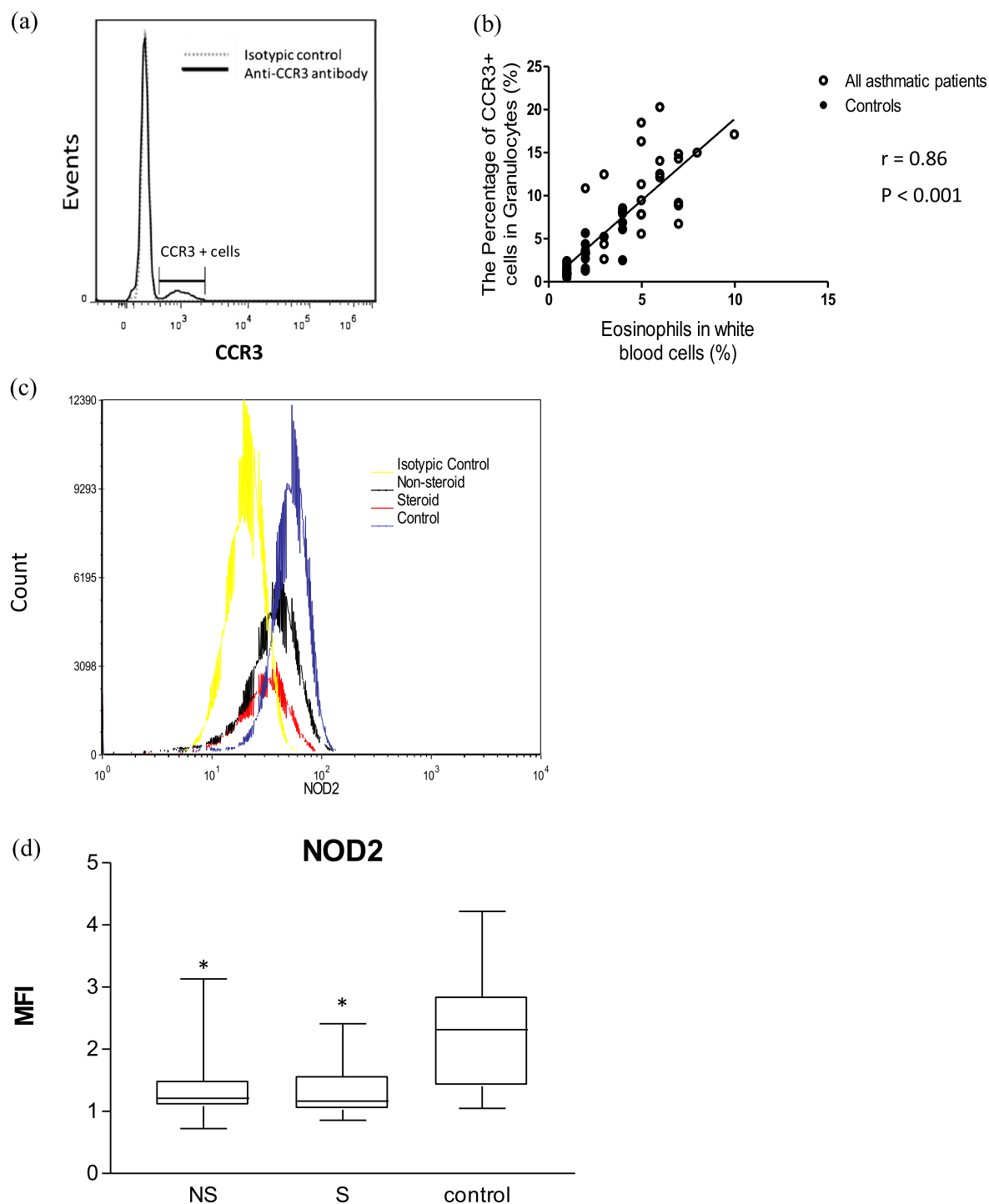


Fig. 2. Expression of NOD2 in CCR3+ granulocytes. **a** Representative histogram is shown for the CCR3+ cells gated from total granulocytes. **b** The correlation between the percentage of CCR3+ cells in granulocytes and the percentage of eosinophils in white blood cells. Correlation was determined by non-parametric Spearman's correlation test. **c** Representative histogram of the intracellular expression of NOD2 in CCR3+ granulocytes is shown. **d** Box and whisker plots showing the comparison of intracellular NOD2 expression (MFI) in CCR3+ granulocytes between asthmatic patients with or without steroid treatment, and control subjects were determined by Mann-Whitney rank sum test. * $p < 0.05$. *Steroid/S* steroid-treated asthmatic patient, *Non-steroid/NS* non-steroid-treated asthmatic patient, *Control* control subject.

Table 2. Correlations Between Different Parameters with DSS of Asthmatic Patients (Values Calculated by Non-Parametric Spearman's Correlation Test. Differences Were Considered Significant when $p < 0.05$)

	Total asthmatic patients	Non-steroid-treated patients	Steroid-treated patients
IL-35	$r=0.037$ $P=0.862$	$r=-0.267$ $p=0.402$	$r=0.577$ $p=0.049^*$
Basogranulin	$r=0.075$ $P=0.729$	$r=-0.531$ $p=0.075$	$r=0.382$ $p=0.220$
Periostin	$r=0.440$ $P=0.031^*$	$r=0.002$ $p=0.994$	$r=0.627$ $p=0.029^*$
NOD2 expression	$r=0.008$ $P=0.974$	$r=0.249$ $p=0.460$	$r=-0.384$ $p=0.218$

* $p < 0.05$

(Fig. 4, $r=-0.648$, $p=0.023$) but plasma IL-35 concentrations did not show any correlation with plasma IL-17A (data not shown, all $p > 0.05$).

In addition, the positive correlation was found between the plasma IL-35 and airway inflammatory cytokine LIGHT in both patients and control subjects (Fig. 5a, b, $r=0.477$, $p=0.019$ and $r=0.554$, $p=0.005$, respectively). Moreover, a relatively low circulating concentration in either LIGHT or its soluble receptor HVEM in the plasma of all control subjects (Fig. 5c) was observed. In the contrast, increased concentration of LIGHT or soluble HVEM was exclusively observed in asthmatic patients with high plasma concentration of IL-35 (>200 ng/mL, $n=7$). In this regard, a negative correlation was found between plasma concentrations of LIGHT and soluble HVEM in these patients with elevated IL-35 plasma concentration ($r=-0.786$, $p=0.036$, Fig. 5d).

DISCUSSION

Dysregulation of cytokines and T cell functions is the immunological feature in the pathogenesis of allergic asthma [8–10, 46]. Imbalance in the functions of Th17 and Treg cells also plays crucial roles in the pathological mechanism in allergic asthma [46, 47]. Although the elevated anti-inflammatory Treg cell cytokines IL-10 and TGF- β have been shown to be necessary for the expression of airway hypersensitiveness and airway remodeling, respectively, in asthma [48, 49], the potential immunopathological roles of novel Treg cell cytokine IL-35 in allergic asthmatic patients have not been extensively studied. This cross-sectional study demonstrated the up-regulation of anti-inflammatory cytokine IL-35 in more severe (steroid-treated) asthmatic patients than controls (Fig. 1). Actually, IL-35 can reverse the IL-17-dependent allergic airway diseases, and attenuate airway inflammation and IgE

production, probably in more severe allergic inflammation [16, 50]. In line with this, we also observe the significant and negative correlation between plasma level of IL-35 and serum total IgE level in asthmatic patient without steroid treatment (Fig. 4). Our present findings of the up-regulation of IL-35 with positive correlation with DSS (Table 2) in severe allergic asthmatic patients may imply the regulatory role of IL-35 being produced in response to the inflammatory reaction in asthma. Plasma IL-17A concentrations were relatively low in this study cohort; therefore, we did not find any significant correlation between IL-35 and IL-17A in groups of patients and controls. However, plasma IL-17A concentrations were found to be significantly higher in asthmatic patients without or with steroid treatment comparing with control group which is in concordance with our previous published results for the activation of Th17 cell in asthma [46]. Further *in vitro* and *in vivo* studies using mice with ovalbumin-induced allergic asthma are on-going to investigate the underlying cellular and intracellular mechanisms of the regulatory roles of IL-35 in allergic asthma.

In allergic asthma, both basophils and eosinophils are effector cells orchestrating allergic inflammation [4]. As shown in Table 1 and Fig. 1, allergic asthmatic patients presented with a higher number of eosinophil count and plasma total IgE concentration, airway eosinophilia marker periostin, and basophil-specific granular protein basogranulin. Basogranulin is secreted from basophils along with histamine in response to IgE-mediated stimulation [51], thereby acting as a valid marker of basophil activation which is distinguishable from mast cell activation [51]. Although basophils were found to be ≤ 1 % in peripheral white blood cells in both allergic asthmatic patients and control subjects, positive BAT upon the challenge by allergens was only found in asthmatic patients but not in control subjects (Table 1). Moreover, plasma basogranulin

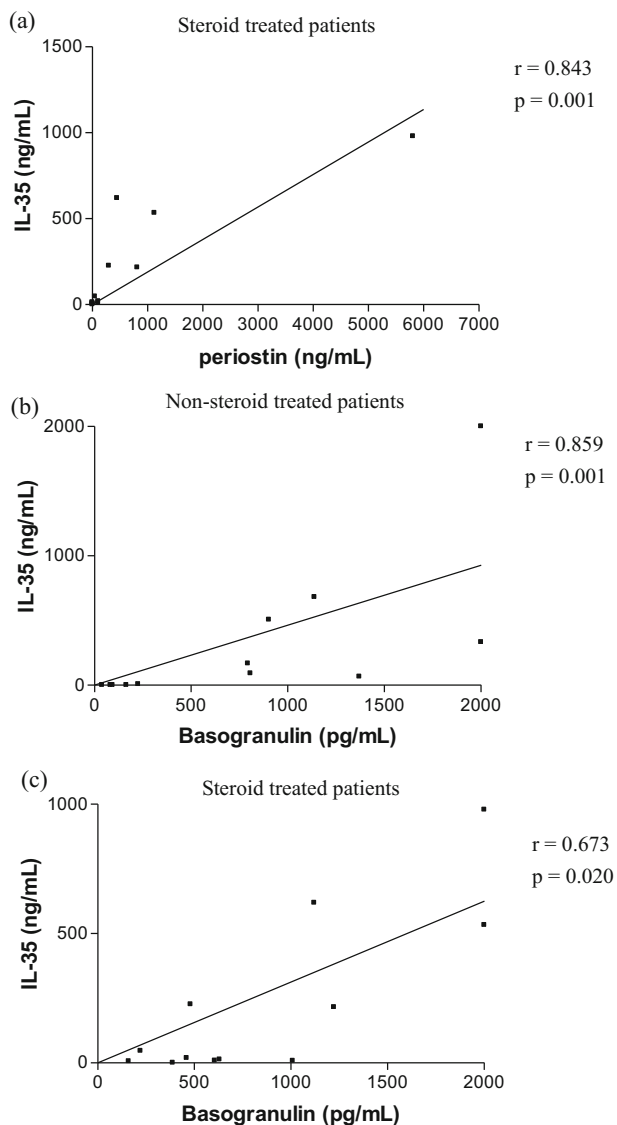


Fig. 3. Correlations between plasma concentrations of IL-35 and periostin of steroid-treated asthmatic patients and IL-35 and basogranulin of allergic asthmatic patients with or without steroid treatment. Correlation was determined by non-parametric Spearman's correlation test.

concentration exhibited positive and significant correlation with plasma concentrations of IL-35 in allergic asthmatic patients with or without the steroid treatment (Fig. 3b, c). Besides, periostin, a systemic matricellular biomarker of airway eosinophilia in asthmatic patients, was shown to be up-regulated in both allergic asthmatic patients with or without steroid treatment (Fig. 1). Periostin also exhibited significant and positive correlation with IL-35 in steroid-treated severe asthmatic patients (Fig. 3a). Results of the elevation of eosinophil

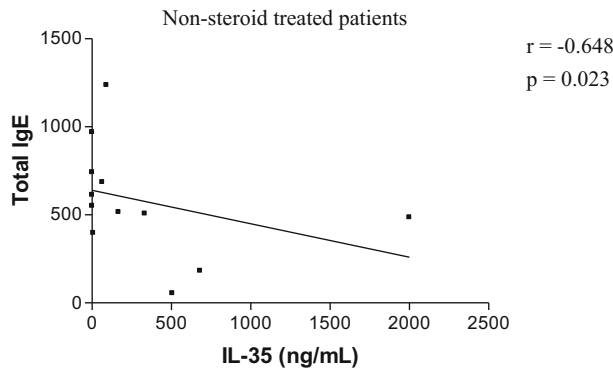


Fig. 4. Correlations among plasma concentrations of IL-35 and total IgE of asthmatic patients without steroid treatment. Correlation was determined by non-parametric Spearman's correlation test.

and basophil markers periostin and basogranulin are therefore in concordance with the positive correlation of IL-35 with DSS. The above results collectively confirmed the integrative roles of iTr35, IgE, eosinophil, and basophil-dependent allergic inflammation and disease severity in allergic asthma.

We have previously demonstrated the expression and activation profile of TLR in allergic asthmatic patients and NOD-mediated activation of eosinophils interacting with bronchial epithelial cells in allergic asthma using *in vitro* and mice model [38, 52]. To further characterize the activation of granulocytes by viral and bacterial infection and innate immunity in allergic asthma, we observed the down-regulation of NOD2 in CCR3+ granulocytes (Fig. 2). Similar to that of our previous study of the expression profile of TLR and downregulation of NOD2 in inflammatory diseases such as asthma and systemic lupus erythematosus [44, 52], our present results also indicated the defective expression of NOD2 in allergic asthma. Results may imply the downregulation or genetic polymorphisms of bacterial sensor NOD2 which may be a predisposition factor for the development of allergic asthma [34, 35]. However, as we did not find any significant correlation of NOD2 expression with IL-35 plasma concentrations (all $p > 0.05$, data not shown), it seems that IL-35 may not have any direct regulatory activity on the NOD2 expression in granulocytes. Further elucidation of the genetic polymorphism, molecular mechanisms for the downregulation of NOD2, intracellular signaling pathways in eosinophils and basophils and the characterization of the expression and function of NOD in other lymphocyte subsets and immune effector cells should be essential to fully elucidate the immunological link between viral and bacterial infections and allergic inflammation.

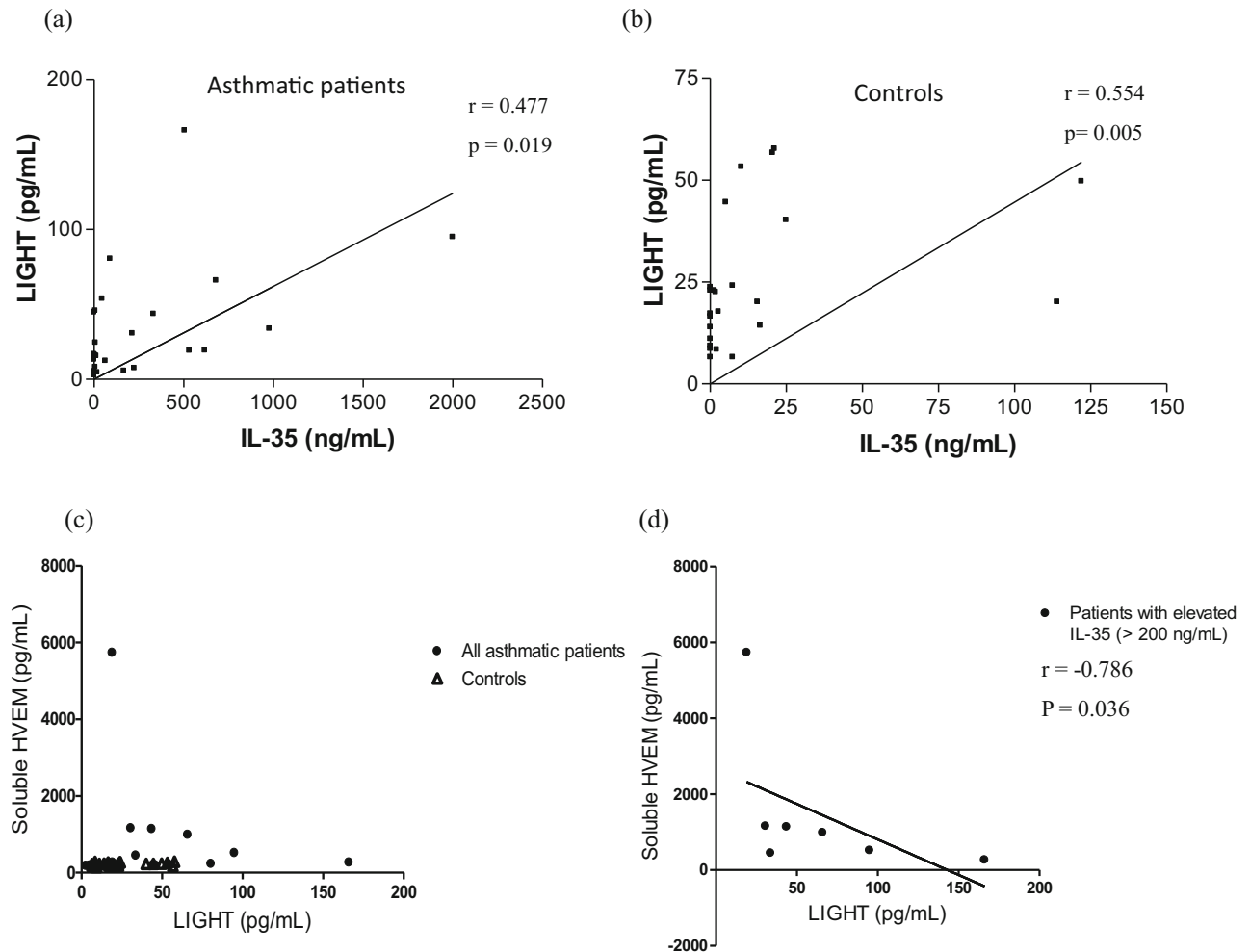


Fig. 5. Plasma concentration of LIGHT and soluble HVEM in allergic asthmatic patients and control subjects. Correlations between plasma concentrations of IL-35 and serum concentrations of LIGHT in **a** asthmatic patients and **b** control subjects were determined by non-parametric Spearman's correlation test. **c** Serum concentrations of LIGHT and plasma concentrations of soluble HVEM of all asthmatic patients and control subjects were determined using ELISA. **d** Correlations between plasma concentrations of LIGHT and soluble HVEM in allergic asthmatic patients with high plasma concentration of IL-35 (>200 ng/mL, $n=7$) were determined by non-parametric Spearman's correlation test.

LIGHT, one of the TNF family members, directly induces airway remodeling, and the pharmacological inhibition of LIGHT using a fusion protein consisting of the IgG Fc domain and LT β R (lymphotoxin β receptor, one of the receptor to LIGHT) can reduce lung fibrosis, smooth muscle/epithelial hyperplasia, and airway hyperresponsiveness in mouse models of chronic asthma [53]. The sputum LIGHT levels in asthmatic patients were also found to correlate with decreased lung function [54]. In the present study, we also observe the significant and positive correlation between plasma IL-35 and LIGHT concentrations in asthmatic patients (Fig. 5a). Although there was no significant difference regarding the

circulating concentrations of LIGHT between asthmatic patients and controls, we observed a negative correlation between LIGHT and soluble HVEM in plasma in asthmatic patients with elevated plasma IL-35 concentration (>200 ng/mL, Fig. 5d). Considering the positive correlation between plasma IL-35 and DSS, asthmatic patients with elevated IL-35 may suffer from a more severe disease severity and inflammatory response. Therefore, the above results may imply that the combined measurement of plasma concentration of LIGHT and soluble HVEM could appropriately reflect the disease severity in severe asthmatic patients with elevated IL-35 regulatory response to inflammation.

CONCLUSION

This clinical study demonstrates the elevation of regulatory cytokine IL-35 in allergic asthma, because of the induction of IL-35 in response to the activation of both eosinophils and basophils in allergic inflammation. IL-35 may serve as a potential surrogate biomarker for disease severity and shed light for development of novel therapeutic modalities for allergic asthma. Since NOD-mediated innate immunity triggered by bacterial infections can be related with allergic inflammation [37, 38], the detailed regulatory mechanisms of IL-35 on the modulation of innate immunity for provoking allergic inflammation in asthma requires further investigations.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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IL-35 and NOD2 in Asthma

- American Journal of Respiratory Cell and Molecular Biology* 44: 127–133.
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