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ORIGINAL ARTICLE

Variation of Regulatory T Lymphocytes in the Peripheral Blood of Children with Allergic Rhinitis

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Abstract

The studies of T-regulatory (Treg) cells in the pediatric allergic disorders, especially allergic rhinitis (AR), are very few and still far from being elucidated. The aim of this study is to assess the frequencies of $CD4^+CD25^+Foxp3^+$ ($CD4^+$Tregs) and $CD8^+CD25^{+high}Foxp3^+$ ($CD8^+$Tregs) regulatory T lymphocytes in the peripheral blood of children with AR. In fresh whole blood of 60 children with AR and 40 healthy controls, the frequencies of $CD4^+$Tregs and $CD8^+$Tregs were examined by flow cytometry. The total IgE concentration in the serum was measured. In AR children, the frequencies of $CD4^+$Tregs and $CD8^+$Tregs were significantly reduced when compared to control group ($p=0.041$, $p=0.011$, respectively). Moreover, the expressions of $Foxp3^+$ in $CD4^+CD25^{+high}$ and $CD8^+CD25^{+high}$ cells were significantly lower in patient group than controls. We found a significant negative correlation between the frequencies of $CD4^+$Tregs and the total IgE concentration ($p<0.01$). In conclusion, the present study demonstrated that the percentages of $CD8^+$Tregs and $CD4^+$Tregs T cells were significantly decreased in children with AR. This suggests that decreased Treg cells might represent a defect in the compartment of T-cell population in children with AR. Further studies are warranted to fully appreciate the clinical relevance of Tregs in children with AR.

Keywords Allergic rhinitis · $CD4^+$Tregs, $CD8^+$Tregs · Children · Regulatory T cells.

Introduction

Allergic rhinitis (AR) is one of the most common allergic disorder of the airway. It is an allergic inflammation of the mucous membranes of the nasal cavity. AR is characterized by mucus hypersecretion, raised serum immunoglobulin E (IgE) and eosinophilic cell infiltration (Okubo et al. 2017; Yu et al. 2017). AR is a type I allergic disease of the nasal mucosa, presented by watery rhinorrhea, repetitive paroxysmal sneezing, and nasal blockage. AR shares the common risk factors of bronchial asthma, particularly atopy (Okubo et al. 2017; Yu et al. 2017). Genetic and environmental risk factors are involved in the pathogenesis of AR; however, the exact etiology still remains to be identified (Turner and Kemp 2012). Currently, AR is a major health problem affecting 10–40% of children globally, which have a great effect on patients' sleep and quality of life, and can affect the school achievement as well as increase the medical costs (Okubo et al. 2017; Turner and Kemp 2012). T lymphocytes are among the main factors that control and organize immune responses in allergic diseases. T-helper (Th)1 T cells release mainly interferon γ and interleukin (IL)-2 and are involved in the delayed hypersensitivity immune reactions, and Th2 T cells release predominantly IL-4 and IL-5 and are predominantly involved in IgE-mediated allergic inflammation. AR is a disease with polarization towards Th2 and a defect of regulatory T cells (Elsayh et al. 2016; Pawankar et al. 2011). $CD4^+CD25^+Foxp3^+$ regulatory T cells ($CD4^+$Tregs) play an important role in the regulation of Th2-induced allergic responses. In addition, they have a major role in preventing organ-specific autoimmunity and allograft rejection, and in maintaining self-tolerance by direct contact with cells and by releasing anti-inflammatory cytokines, e.g., IL-10 (Hsu

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et al. 2010; Yu et al. 2017). $CD4^+$Tregs preserve a state of tolerance to the harmless substances and limit improper or excessive immune responses. $CD4^+$Tregs control the development of allergic reactions by numerous pathways. They block the migration of effector T cells into the inflamed tissue, inhibit activation of Th2 cells, suppress the production of IgE, and induce IgG4 in B cells (Stelmaszczyk-Emmel et al. 2013; Yu et al. 2017; Zahran et al. 2016). Recent studies have reported that the levels of $CD4^+$Tregs were significantly low in children with allergic diseases (Lee et al. 2007; Shaoqing et al. 2016). Recently, few studies have started to investigate the role of $CD4^+$Tregs in the course of allergy and the link between this subpopulation and immunotherapy (Yu et al. 2017). $CD8^+CD25^{+high}Foxp3^+$ cells ($CD8^+$Tregs) are another important population of Treg cells. Nevertheless, few studies characterized $CD8^+$Treg cells. They block the activation of naive T lymphocytes and suppress the proliferation of $CD4^+$ T lymphocytes (Eusebio et al. 2014; Tsai et al. 2010). The studies of Tregs in the pediatric allergic disorders, especially AR, are very few and still far from being elucidated. The aim of this study is to assess the frequencies of $CD4^+CD25^+Foxp3^+$ and $CD8^+CD25^{+high}Foxp3^+$ regulatory T lymphocytes in the peripheral blood of children with AR.

Materials and Methods

Compliance with Ethical Standards

The procedures of this study were in accordance with the regulations of the relevant clinical research ethics committee and with those of the code of ethics of the world medical association Declaration of Helsinki. Written informed consents of legal caregivers of all children were taken after explanation of the study objectives and its benefits for their children. Faculty of Medicine, Assiut University Ethical Committee approved the study protocol.

Study Design

This was a case-controlled study undertaken in Assiut and Al-Azhar University Hospitals, Assiut city, Egypt.

Patients

This study included that sixty patients aged 6–13 years with AR were recruited to the study from two outpatient clinics in Al-Azhar and Assiut University Hospitals, Assiut, Egypt during winter 2016 (from September to December). The diagnosis of AR was performed according to Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines (Brozek et al. 2010) by a senior otorhinolaryngologist before the patients were recruited into the study. Classification and severity grading of patients were performed based upon ARIA criteria such as intermittent (mild or moderate–severe) or persistent (mild or moderate–severe) rhinitis (Brozek et al. 2010). Forty age- and sex-matched non-atopic, non-asthmatic healthy children were recruited as normal control subjects. Any treatment with systemic corticosteroids and antihistamines was stopped 4 weeks before the study. The exclusion criteria were: (1) manifestations of exacerbation of the allergic symptom; (2) accompanying other diseases, e.g., allergic asthma, deviated nasal septum, sinusitis, and acute respiratory tract infections; (3) recent history of immunotherapy; and (4) patients with rhinitis of infectious and/or inflammatory, occupational, drug-induced, hormonal, or other non-allergic causes were excluded.

Laboratory Investigations

All participants underwent laboratory tests which included complete blood picture with differential count. The levels of total IgE were measured by ELISA. All samples were measured in duplicate, in addition to flow cytometric detection of Treg cells, B and T lymphocytes.

Flow Cytometric Detection of Treg Cells, B and T Lymphocytes

Treg cells in whole blood samples were enumerated using fluoroisothiocyanate (FITC)-conjugated Foxp3 (eBioscience, USA), phycoerythrin (PE)-conjugated CD25 (IQ Product, The Netherlands), peridinium–chlorophyll–protein (Per-CP)-conjugated CD4, and allophycocyanin (APC)-conjugated CD8 (Becton Dickinson, Bioscience, USA). 50 µl of blood sample was incubated with 10 µl of CD4, CD8, and CD25 for 15 min at room temperature in the dark. Following incubation, red blood cell lysis, washing with phosphate buffer saline (PBS), addition of fixed solution to fix the cells, and incubation for 10 min were done. After incubation, cells were washed with PBS, and then permeabilized solution and 10 µl of Foxp3 were added and incubated for 30 min at room temperature. For detection of B lymphocytes and T lymphocytes, 50 µl of blood sample was stained with 10 µl of FITC-conjugated CD4, PE-conjugated CD8 and Per-CP-conjugated CD3, and APC-conjugated CD19 in another tube, all from Becton Dickinson Biosciences, USA. The tubes were incubated for 15 min at room temperature in the dark PBS. Flow cytometric analysis was done by FACSCalibur flow cytometry with the CellQuest software (Becton Dickinson Biosciences, USA). Anti-human IgG was used as an isotype-matched negative control for each sample. Forward and side scatter histogram was used to define the lymphocyte population (R1). Then, the percentages of $CD19^+$ (B lymphocytes), $CD3^+$ (T lymphocytes), $CD4^+$ (Th

cells), and $CD8^+$ (T cytotoxic) were assessed in lymphocyte population.

Total $CD4^+CD25^+$, $CD4^+CD25^{+intermediate\ (low)}$, and $CD4^+CD25^{+high}$ defined as the population of CD4 positive T cells, whose CD25 expression exceeded the level of CD25 positivity seen in the CD4 negative T cells and $CD4^+CD25^{+high}Foxp3^+$ Treg cells, were evaluated as a percentage of $CD4^+$. In addition, total $CD8^+CD25^+$, $CD8^+CD25^{+intermediate(low)}$, and $CD8^+CD25^{+high}$ and $CD8^+CD25^{+high}Foxp3^+$ Treg cells were evaluated as a percentage of $CD8^+$. The expression of $Foxp3^+$ in $CD4^+CD25^{+high}$ and in $CD8^+CD25^{+high}$ cells was expressed as geometric mean of fluorescence intensity, as shown in Fig. 1.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD), by the statistical package for social sciences SPSS 22 (SPSS Inc., USA). Flow cytometric data were compared statistically between the groups by using the Mann–Whitney *U* test. The correlation coefficient *r* was generated by Spearman's rank correlation. Statistical significance was defined as $p < 0.05$.

Results

As shown in Table 1, the study participants in AR patients and control groups were comparable for anthropometric data, age, and gender. AR children had total serum IgE values significantly higher than those of control group ($p < 0.001$). Table 2 shows clinical data and classification of patient group.

The frequencies of total lymphocytes, T lymphocytes, and $CD8^+$ were significantly higher in patients with AR in comparison with controls ($p = 0.004$, $p < 0.0001$, and $p = 0.001$, respectively; Table 2), while there was no significant difference in $CD4^+$ and B lymphocytes ($p = 0.166$ and $p = 0.184$, respectively). The frequency of $CD4^+CD25^{+high}$ and $CD8^+CD25^{+high}$ T cells was significantly reduced in patients with AR in comparison with controls ($p = 0.02$, $p = 0.001$, respectively; Table 3), while there were no significant differences in total $CD25^+CD4^+$, $CD4^+CD25^{+low}$, total $CD25^+CD8^+$, and $CD8^+CD25^{+low}$ populations between the patients and healthy children. The proportion of $CD4^+CD25^{+high}Foxp3^+$ cells was significantly reduced when compared to the control group ($p = 0.041$; Table 3). Moreover, the expression of $Foxp3^+$ in $CD4^+CD25^{+high}$ cells was significantly lower in patient group when compared to control ($p = 0.007$; Table 3). The proportion of $CD8^+CD25^{+high}Foxp3^+$ cells and the expression of $Foxp3^+$ in $CD8^+CD25^{+high}$ cells were significantly reduced when compared to the control group ($p = 0.011$ and $p = 0.001$, respectively; Table 3). Finally, there was a significant negative correlation between the number of $CD4^+CD25^{+high}Foxp3^+$ cells and the total IgE concentration ($r = -0.72$, $p < 0.01$).

Discussion

In our study, the frequencies of circulating $CD4^+CD25^{+high}Foxp3^+$ ($CD4^+$Tregs) and $CD8^+CD25^{+high}Foxp3^+$ ($CD8^+$Tregs) T-regulatory lymphocytes in children with AR were analyzed. The findings of our study could demonstrate that the frequencies of $CD4^+$Tregs and the expression of $Foxp3^+$ in $CD4^+CD25^{+high}$ cells were lower in AR children compared to age-matched non-allergic healthy controls. The differences were statistically significant in a relatively large number of allergic children. This suggests that the reduced effect of Tregs may have a role in the pathogenesis of AR in children.

Recently, different studies have started to find an association between allergic disorders and defective immunoregulation; however, the results of researches were inconsistent. The role of Tregs in the pathogenesis of AR and atopy was not defined until the last decade. Patients with deficiency of $CD4^+CD25^+$ Tregs [such as patients with a syndrome of systemic autoimmunity caused by Foxp3 mutations (IPEX)] presented with food allergy, eczema, high IgE levels, and eosinophilia (Gambineri et al. 2003). $CD4^+$Tregs have different effects in allergic disorders. This population may block the activation of Th2, decrease allergic inflammation of the airway, and can stop the inappropriate Th2 response to innocuous allergens (Kearley et al. 2005; Lee et al. 2007). Previous independent lines of evidence indicated that the number and/or function of $CD4^+$Tregs are altered in patients with allergies when compared with healthy controls (Geraldes et al. 2010; Grindebacke et al. 2004; Lee et al. 2007; Ling et al. 2004; Mészáros et al.2009; Shaoqing et al.2016; Stelmaszczyk-Emmel et al. 2013; Sun et al. 2016; Xu et al. 2007; Zhang et al. 2008). However, the results of these studies in allergic patients have yielded contradictory outcomes. In addition, the data regarding the role of Tregs in the pathogenesis of allergic disease in children are rare.

In line with our results, the first group of researches (Lee et al. 2007; Shaoqing et al. 2016; Sun et al. 2016; Stelmaszczyk-Emmel et al. 2013; Xu et al. 2007) has reported a reduction of the $CD4^+$Tregs compartment in patients with allergic disorders. Moreover, Lee et al. (2007) and Sun et al. (2016) have reported that $CD4^+CD25^+$ T cells were significantly lower in numbers in pediatric patients with airway allergy when compared to healthy controls. Zhang et al. (2008) found a significantly decreased number of $CD4^+$Tregs only in patients with acute exacerbation of asthma in comparison

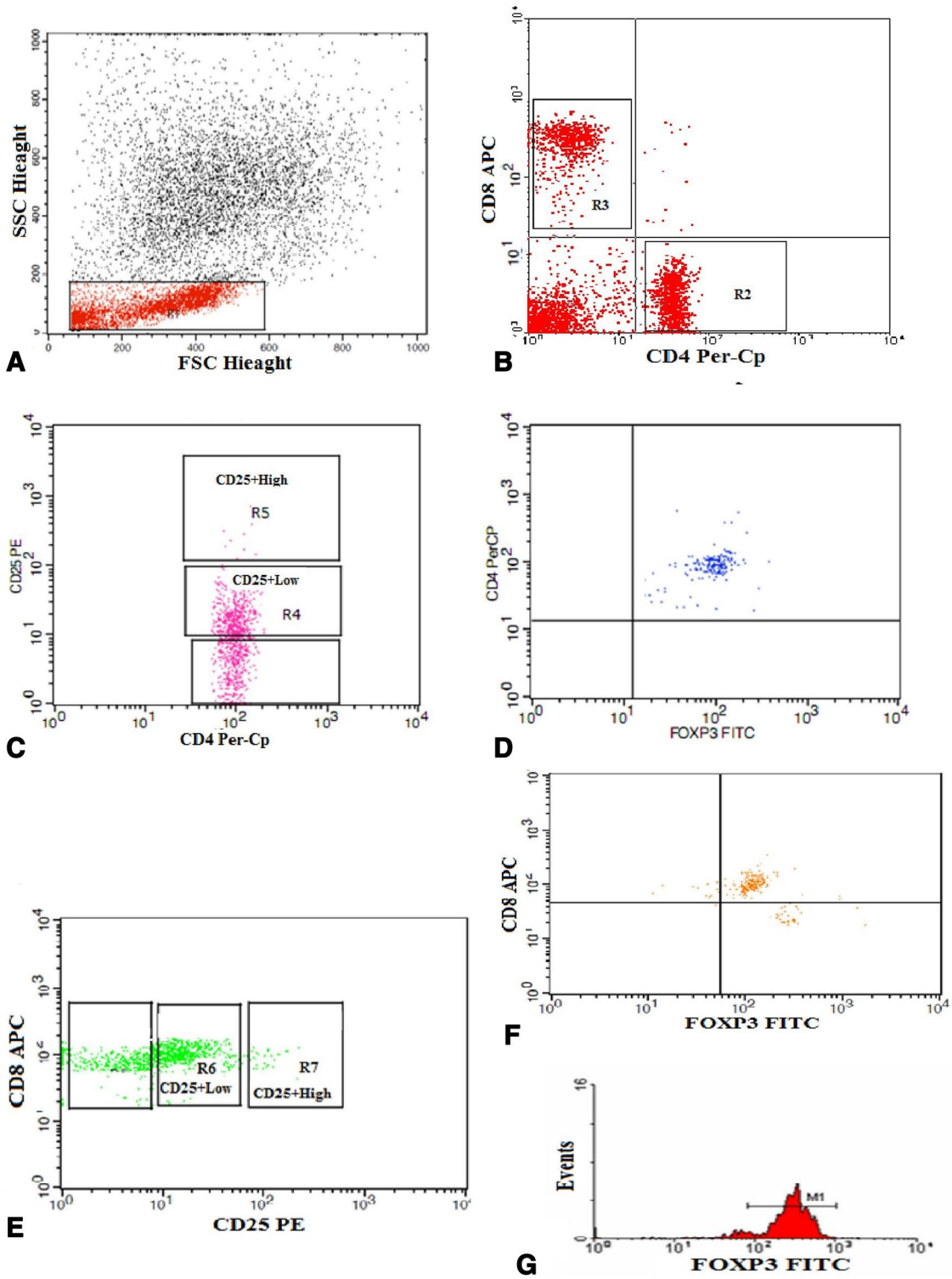

Fig. 1 Flow cytometric detection of regulatory T cells **a** Scatter histogram was used to define the lymphocyte population (R1). **b** $CD4^+$ (R2) and $CD8^+$ (R3) cells were detected on lymphocyte population. **c** The expression of CD25 on $CD4^+$ then detected and different gates were drown to define $CD4^+CD25^{+low}$ cells (R4), and $CD4^+CD25^{+high}$ cells (R5). **d** Then the percentage of $CD4^+CD25^{+high}$ $FoxP3^{+cells}$ ($CD4^+$ regulatory T cells) was determined. **e, f** Show the detection of $CD8^+$ regulatory T cells on $CD8^+$ cells. The expression of CD25 on $CD8^+$ detected. Then $CD8^+CD25^{+low}$ cells (R6), and $CD8^+CD25^{+high}$ (R7) cells and $CD8+CD25^{+high}$ $FoxP3^{+cells}$ ($CD8^+$ regulatory T cells) were determined. **g** Show the expression of $FoxP3^+$ on $CD8+CD25^{+high}$ cells

Table 1 Demographic and clinical characteristics of study participants

	AR patients ($n = 60$)					Controls ($n = 40$)					p value
	Mean	SD	Median	Min	Max	Mean	SD	Median	Min	Max	
Age	7.6	2.4	8.1	5.1	13.2	8.0	1.9	8.7	5.0	13.5	NS
Weight	28.5	13.0	29.5	15.8	60.8	29.3	13.1	28.8	16.1	70.2	NS
Height	124.0	15.0	135.0	106.0	153.0	123.0	14.0	134.0	102.0	151.0	NS
BMI	17.7	4.6	16.7	14.4	29.8	17.5	4.2	16.5	13.1	30.5	NS
Gender: number (%)											
Males	33 (55)					22 (52)					NS
Females	27 (45)					18 (45)					NS
Total IgE (IU/ml)	136.5 ± 41.3					42.8 ± 15.9					< 0.001*

*Significant, *NS* non-significant

Table 2 Classification and clinical data for AR patients

ARIA classification:	Number (%)
Moderate/severe intermittent	10 (16.6)
Mild persistent	28 (46.7)
Moderate/severe persistent	22 (36.7)
Clinical manifestations	Number (%)
Rhinorrhea	57 (95.0)
Sneezing	58 (96.7)
Nasal obstruction	49 (81.7)
Nasal itching	48 (80.0)

with stable asthmatics and healthy controls. The second group of researches (Geraldes et al.2010; Grindebacke et al.2004; Ling et al. 2004; Mészáros et al. 2009) stated that the frequency of $CD4^+$Tregs was comparable between allergic patients and healthy non-allergic controls. Nevertheless, the third group of researchers reported higher frequencies of $CD4^+$Tregs in atopic patients when compared with controls (Karagiannidis et al. 2004; Shi et al. 2004). The explanation given for this discrepancy of our results and these studies remains hypothetical; however, it may be related to different age and number of the patients, the clinical type of the allergic disease, severity, and disease status. Moreover, the conflicting results may depend on the way of detecting Tregs populations. Not all studies defined $CD4^+$Tregs as $CD4^+CD25^{high}Foxp3^+$ T cells. In certain analyses, $CD4^+$Tregs were defined as $CD4^+CD25^{high}$ T cells. Moreover, the type of analysis can be influenced by

Table 3 Lymphocyte population in AR patients and controls

Percentage %	Patients ($n = 60$)	Control ($n = 40$)	p value
Total lymphocytes	56.85 ± 10.96	44.57 ± 12.83	0.004
B lymphocytes	13.82 ± 12.14	15.22 ± 1.71	0.184
T lymphocytes	66.04 ± 9.92	47.95 ± 9.2	< 0.0001
$CD8^+$T lymphocytes	38.1 ± 7.6	21.4 ± 5.8	0.001
$CD4^+$T lymphocytes	54.65 ± 15.19	49.85 ± 10.19	0.166
CD4/CD8 ratio	1.47 ± 0.51	1.91 ± 0.51	0.030
$CD25^+CD4^+$	7.57 ± 0.28	8.04 ± 0.21	0.366
$CD4^+CD25^{+low}$	4.95 ± 0.12	5.18 ± 0.12	0.423
$CD4^+CD25^{+high}$	1.59 ± 0.06	2.27 ± 0.53	0.020
$CD4^+CD25^{+high}Foxp3^+$	1.4 ± 0.19	1.69 ± 0.37	0.041
MFI of $Foxp3^+$ expression in $CD4^+CD25^{+high}$	102.69 ± 17.07	121.47 ± 35.17	0.007
$CD25^+CD8^+$	6.71 ± 0.45	7.96 ± 0.75	0.197
$CD8^+CD25^{+low}$	4.68 ± 0.44	5.01 ± 0.84	0.989
$CD8^+CD25^{+high}$	2.07 ± 0.13	2.99 ± 0.21	0.001
$CD8^+CD25^{+high}Foxp3^+$	1.07 ± 0.14	1.68 ± 0.19	0.011
MFI of $Foxp3^+$ expression in $CD8^+CD25^{+high}$	96.24 ± 27.33	112.94 ± 26.34	0.001

Data represented as means ± SD

Mann–Whitney Test

Foxp3 forkhead box protein 3, *MFI* mean fluorescent intensity

$p \leq 0.05$ is significant

different laboratory techniques, time and season of specimen collection, and types and doses of drug regimens of patients enrolled in studies, etc. Yet, most of the previous researches agreed that $CD4^+$Tregs are downregulated in patients with allergic disorders and their conclusions were in line with our findings. As regards to AR, the previous studies showed decreased Foxp3 mRNA expression in the mononuclear cells in nasal secretions, nasal tissue, and peripheral blood of patient with AR (Lee et al. 2009; Xu et al. 2007) suggesting a substantial role of $CD4^+$Tregs in reducing the allergic response.

The association between allergic disease severity and the proportion of $CD4^+$Tregs has been reported in few studies (Lee et al. 2007; Mészáros et al. 2009; Shi et al. 2004). These studies demonstrated that the frequencies of $CD4^+$Tregs increased during the exacerbation of asthma (Shi et al. 2004) and in severe asthma (Lee et al. 2007; Mészáros et al. 2009). As regards AR, Lee et al. (2007) reported that children with persistent AR had significantly higher frequencies of $CD4^+$Tregs when compared to patients with intermittent AR. However, we observed no significant association between AR severity and the frequencies of $CD4^+$Tregs in our study.

We found a significant negative correlation between the frequencies of $CD4^+$Tregs and the total IgE concentration ($p < 0.01$). This result is consistent with the previous reports (Stelmaszczyk-Emmel et al. 2013; Shaoqing et al. 2016) which indicated that there was a significant correlation between $CD4^+$Treg percentage and IgE serum concentration. In contrast to our result, Lee et al. (2007) and Hartl et al. (2007) reported that the number of $CD4^+$Tregs was not correlated with total serum IgE level.

In the present study, our results showed for the first time that the percentages of $CD8^+$Tregs and the expression of $Foxp3^+$ in $CD8^+CD25^{+high}$ cells were significantly lower in children with AR when compared to age-matched non-allergic control children. $CD8^+CD25^{+high}Foxp3^+$ cells ($CD8^+$Tregs) are emerging as an important subset of Treg cells. However, $CD8^+$Tregs are not well characterized as $CD4^+$Tregs. $CD8^+$Tregs block the activation of naive T lymphocytes and suppress the responses of IgG and IgE antibodies. Furthermore, $CD8^+$Tregs suppress the proliferation of $CD4^+$ T lymphocytes and IL-4 (Eusebio et al. 2014, 2015). While most of the previous researches were focusing only on $CD4^+$Treg functions, large-scale studies on the role of $CD8^+$Tregs population in allergic disorders are scarce (Eusebio et al. 2015). Eusebio et al. (2014, 2015) reported that $CD8^+$Tregs were significantly decreased in blood of asthmatic patients when compared to healthy control. Furthermore, the levels of Foxp3 mRNA $CD8^+$ T cells were significantly lower in severe asthma in comparison with mild-to-moderate asthma and control patients. $CD8^+$Treg population was first recognized in human tonsils; upon invitro activation, $CD8^+Foxp3^+$Tregs can inhibit T-cell proliferation directly (Siegmund et al. 2009). $CD8^+$Tregs also have a regulatory function that decreases autoimmune disorders in experimental animals (Tsai et al. 2010). In mice, systemic allergen immunization induces $CD8^+$Tregs cells, which can inhibit the development of allergic diarrhea (Yamada et al. 2009).

$CD8^+$Tregs produce their suppressive function by direct killing of the target cells by cell-to-cell contact or by secretion of different cytokines, including transforming growth factor (TGF)-β and IL-10 which shift the production of antibodies from IgE to non-inflammatory IgA and IgG4 and inhibit inflammation in the airways (Eusebio et al. 2011). The implication of $CD8^+$Tregs in the pathogenesis of asthma developed from the collaboration with $CD4^+$Tregs. $CD4^+$Tregs apply their suppressive functions partly through their cytokines, namely, IL-10 and TGF-β. These cytokines are also secreted by $CD8^+$Tregs. The suppressor mechanism of $CD4^+$Tregs relays in association with $CD8^+$Treg population. Due to the resemblances in the cytokine profile of $CD8^+$Treg and $CD4^+$Treg T cells and their mutual cross-talking, the implication of $CD8^+$Tregs in the pathogenesis and control of allergic inflammation is logical (Eusebio and Pietruczuk 2011).

In conclusion, the present study demonstrated that the percentages of $CD8^+$Treg and $CD4^+$Treg T cells were significantly decreased in children with AR. This suggests that decreased Treg cells might represent a defect in the compartment of Treg-cell population in children with allergic AR. Further studies are warranted to fully appreciate the clinical relevance of Tregs in children with AR.

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Compliance with ethical standards

Conflict of interest All authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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