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Comparison of the Percentage of Regulatory T cells and their p-STAT5 Expression in Allergic and Non-Allergic Common Variable Immunodeficiency Patients

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ABSTRACT

Background: Common Variable Immunodeficiency (CVID) is a primary immunodeficiency characterized by an immunologic deficiency in immunoglobulin production. Regulatory T cells (Tregs) play a key role in preventing the development allergic disorders. p-STAT5 is a known factor for the function and survival of Tregs. This study aimed to investigate the number of Tregs and their p-STAT5 expression in allergic and non-allergic CVID patients.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from 10 healthy volunteers, 10 allergic patients, and 16 CVID patients (allergic and non-allergic) using Ficoll density centrifugation. The percentage of Tregs in PBMCs was analyzed by flow cytometry. Tregs were also isolated from participants using an immunomagnetic separation method and p-STAT5 expression was evaluated in Tregs using flow cytometry.

Results: The results revealed that Treg percentage was significantly lower in the CVID patients than the control groups (healthy and allergic individuals) ($p<0.001$). There was a significant reduction in Treg percentage in allergic patients compared to healthy subjects ($p<0.05$). No significant difference in Treg percentage between allergic and non-allergic CVID patients was observed. The expression of p-STAT5 in Tregs was significantly lower in CVID patients than the control groups ($p<0.001$). In addition, the expression of p-STAT5 in Tregs of allergic patients was significantly decreased compared to healthy subjects ($p<0.001$). However, the deference of p-STAT5 level was not statistically significant between allergic and non-allergic CVID patients.

Conclusion: These findings suggest that p-STAT5 signaling defect and decreased Treg number may not participate in the development of allergy in CVID patients.

Keywords
Allergy; common variable immunodeficiency; p-STAT5 protein; regulatory T cells

Introduction

Common variable immunodeficiency (CVID) is the most frequent symptomatic primary immunodeficiency characterized by impaired antibody responses, recurrent bacterial infections, and low levels of serum IgG, IgA, and/or IgM (Cunningham-Rundles and Bodian, 1999; Egawa et al., 2018; Grace et al., 2009). Although the most important

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immunologic impairment is the lack of immunoglobulin production by B cells, in a small proportion of CVID patients, defects in the number and function of T lymphocytes have been reported (Arumugakani et al., 2010; Sadeghi et al., 2015). The etiology of CVID remains unknown, although mutations in ICOS, CD19, BAFF-R, and TACI genes have been implicated (Fleischman et al., 1998). These patients usually suffer from recurrent respiratory infections and have the elevated incidence of autoimmune, lymphoproliferative granulomatous, and gastrointestinal diseases (Melo et al., 2009). Moreover, allergic disorder is a major problem observed in some of patients with CVID (Agondi et al., 2010; Özcan et al., 2014). Clinical studies have shown that CVID patients are more susceptible to allergic disorders such as atopy and asthma than healthy subjects (Agondi et al., 2010). In addition, it has been shown that there is a bias from Th1 immunity towards Th2-type responses in CVID patients (Rezaei et al., 2008a). In subjects with CVID, allergic immune responses are initiated by Th2 cell activation and its cytokines production. In turn, cytokines induce the production of allergen's specific IgE, leading to liberation of active chemicals from different immune cells and subsequently symptoms of allergic diseases (Williams et al., 2012).

A key component in the maintenance of self-tolerance is regulatory T cells (Tregs) (Arumugakani et al., 2010; Brusko et al., 2007). They constitute about 5–10% of the peripheral blood $CD4^+$ T cells and have an indispensable role in preventing the development of autoimmune and allergic disorders (Genre et al., 2009; Grace et al., 2009; Nava et al., 2009). Extensive data from the literature have reported that the reduced number and impaired function of Tregs are largely associated with allergic disorders and autoimmune diseases (Larche, 2007; Palomares et al., 2010). A great number of studies have been carried out on the role of Tregs in preventing the development of allergy in patients suffered from allergic disorders (Lesiak et al., 2012; Wu et al., 2008). Immunomodulatory effects of Tregs on the prevention of allergic reactions are mediated by various mechanisms including: I) suppression of Th2-type responses to allergen, II) inhibition of the activation of eosinophil, mast cell and basophil and change of antibody isotype from IgE to IgG4, III) reduction of dendritic cell function and immune cell migration to tissues (Larche, 2007; Özcan et al., 2014).

Several lines of evidence show that signal transducer and activator of transcription (STAT) protein 5 plays a fundamental role in survival, proliferation, and suppressive functions of Tregs (Grace et al., 2009). STAT5 function is mediated by phosphorylation of this protein by kinases associated with transmembrane receptors (Grace et al., 2009). The defects in the phosphorylation and function of STAT5 participate in the reduced number and impaired function of Tregs which are associated with the risk of developing autoimmune and allergic diseases (Nadeau et al., 2011).

Previous studies investigated the correlation between the reduction of Treg number with chronic inflammation, splenomegaly, and autoimmune manifestations in CVID patients (Arandi et al., 2013; Arumugakani et al., 2010; Horn et al., 2009; Nadeau et al., 2011). This study is aimed to investigate and compare the percentage of Tregs and their p-STAT5 expression level in CVID patients with allergic and without allergic symptoms, allergic patients, and healthy subjects.

Table 1. Specifications of the subjects studied.

	Patient groups (n = 16)		Control groups (n = 20)	
	Allergic CVID (n = 8)	Non-Allergic CVID (n = 8)	Allergic patient group (n = 10)	Healthy individual (n = 10)
Sex (male/female)	5/3	4/4	5/5	6/4
Age (mean ± SD)	19.75 ± 4.26	22 ± 1.48	29.2 ± 5.28	22.4 ± 5.17

Materials and methods

Subjects

A total of 36 individuals were recruited among those referred to the immunodeficiency clinic of Alzahra hospital, Isfahan, Iran from September 2016 to March 2017 (Table 1). CVID was diagnosed by specialist according to the European Society for immunodeficiency (ESID) criteria including: a reduction in the serum antibody level of at least two isotypes of IgG, IgM or IgA, Onset of immunodeficiency at greater than two years of age, absence of isohemagglutinin, a poor response to vaccination, and the rejection of other causes of hypogammaglobulinemia (Agata, et al.). Since all patients were treated with intravenous immunoglobulin (IVIG), the sampling was performed after 3–4 weeks of IVIG injection (before the next injection). None of CVID patients were on treatment with a drug known to affect the immune system and antibodies production (i.e. steroids, phenytoin, sulfasalazine, antimalarial drugs, and gold salts) at the time of the blood draw and all of the patients are currently alive. Allergy diagnostic were performed by specialist using clinical and laboratory diagnostic criteria such as serum IgE measurement and skin prick test. A questionnaire was used to the patients prospectively pertaining to their food allergies.

Antibody assay

To evaluate the immunologic situation of patients and affirm CVID disease, the serum levels of IgA and IgM in healthy volunteers, CVID, and allergic patients were measured using an Enzyme-linked immunoasorbent assay (ELISA) kit according to the manufacturer's protocol (Mabtech, Sweden). The serum levels of IgA and IgM in healthy volunteers and allergic patients were considered as control groups.

Isolation of peripheral blood mononuclear cells (PBMCs)

Heparinized blood samples (10 ml) were obtained from participants and PBMCs were isolated using Ficoll density centrifugation based on the manufacturer's instructions (Miltenyi Biotec, Germany). Informed consent for participation in the study was obtained from contributors before entering the study. The study and all protocols were approved by the Ethics Committee of Isfahan University of Medical Sciences (reference number: 394637) and performed in accordance with the declaration of Helsinki. After centrifugation, PBMCs were collected from the interface between Ficoll and the plasma and washed twice with phosphate buffered saline (PBS). PBMCs were suspended in PBS and cell viability was determined using trypan blue dye exclusion. Cell count was also performed with a haemocytometer.

Tregs staining

The isolated PBMCs (1×10^6 cell/ml) were stained by regulatory T cell staining kit (eBioscience, USA). Accordingly, the cells were stained with fluorescein isothiocyanate (FITC) anti-human CD4 (eBioscience, USA) and Phycoerythrin (PE) anti-human CD25 antibodies (eBioscience, USA) or the matched isotype control IgG for 25 min at 4°C. The matched isotype control antibodies were used as negative controls. After fixation and permeabilization with Fix/Perm buffer (eBioscience, USA), the cells were stained intracellularly with Phycoerythrin/Cyanine5 (PE/cyn5) anti-human Foxp3 antibody (eBioscience, USA) for 25 min at 4°C. Afterwards, the cells were washed three times with PBS. Data were obtained using a FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA) and analyzed by FlowJo software (v10.1, FlowJo, Ashland, OR, USA). The $CD4^+$, $CD25^+$ and $Foxp3^+$ cells were considered as Tregs.

Purification of Tregs from peripheral blood

$CD4^+$ $CD25^+$ cells were isolated by a magnetic cell sorting (MACS) method using human $CD4^+$ $CD25^+$ regulatory T cells isolation kit (Miltenyi Biotec, Germany). Following the manufacturer's protocol, two-step procedure was used to isolate Tregs. Briefly, $CD4^+$ T cells were first isolated from PBMCs using an indirect magnetic labeling of non $CD4^+$ T cells (negative selection) with biotin-conjugated monoclonal antibodies cocktail and anti-biotin Micro Beads. Afterwards, a positive selection of $CD25^+$ T cells was preformed following a direct magnetic labeling of $CD25^+$ T cells with anti-CD25 Micro Beads. To determine the purity of $CD4^+CD25^+$ Foxp3+ Tregs, cells were stained with FITC anti-human CD4 and PE anti-human CD25 antibodies or the matched isotype control IgG for 25 min at 4°C. The relevant isotype control antibodies served as negative controls. Fixation and permebilization of the cells were carried out using Fix/perm buffer and then subjected to intracellular staining using PE/cyn5 anti-human Foxp3 or the relevant isotype control antibodies according to the manufacturer's instructions (eBioscience, USA). All antibodies and buffers were purchased from eBioscience (USA). The purity of cells was analyzed using a FACSCalibur flow cytometer. Cell samples with a > 92% purity were used in the following experiments (data not shown).

Assessment of p-stat-5 expression level in Tregs

Tregs (5×10^5 cell/ml) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera, France) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Biosera, France) at 37°C and 5% CO_2 for 4 hours. Afterwards, the cells were stimulated with interleukin-2 (500 U/ml). After 15 min of incubation at 37°C, intracellular (IC) fixation buffer was used to fix the cells in the dark for 45 min according to the manufacturer's protocol (eBioscience, USA). The fixed cells were then permeabilized by methanol for 45 min at 4°C based on the manufacturer's instructions (eBioscience, USA). Intracellular staining was performed with PE anti-human/mouse p-STAT5 antibody (eBioscience, USA) for 45 min at 4°C and the stained cells were washed three times with PBS. The percentage and mean fluorescence intensity (MFI) of the stained cells were measured using a FACSCalibur flow cytometry and then analyzed using FlowJo software.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). The results were analyzed using SPSS (V. 19, IBM, Chicago, IL.). Comparison between two groups with normal and non-normal distribution was performed using Student's t-test and Mann-Whitney U test, respectively. p value < 0.05 was considered statistically significant.

Result

Description of subjects

16 CVID subjects (aged 20.88 ± 8.8, mean ± standard deviation, range: 9 to 39 years) were enrolled in the study. The onset of clinical symptoms in patients was varied and occurred from an age of 2 years old to 20 (Table 2). The age in which the illness was diagnosed varied from a range of 2 years old to 25 (data not shown). Eight patients had various infections early from the birth as a consequence of consanguineous marriages. The most common clinical manifestations among CVID patients were allergies, respiratory infection, and middle ear infection. Of the 16 CVID patients, eight had allergic symptoms, while eight did not. Four CVID patients with allergic symptoms had frequent middle ear infection, two had respiratory infection, lymphadenopathy, lymphohyperplasia, and joint's pain, and 1 had bronchiectasis, seizure, brain encephalitis, weight loss and hyperplasia (Table 2). Of the eight non-allergic CVID patients, six had frequent middle ear infection, three had bronchiectasis, two had respiratory infection, and 1 had lymphadenopathy, chronic diarrhea, joint's pain, nephritic syndrome, brain encephalitis, meningitis, pneumonia, and malignant history (Table 2). Table 2 depicted demographics and the clinical characteristics of CVID subjects.

Immunologic properties of CVID patients

Since CVID disease is mainly associated with immunologic impairments in immunoglobulin production by B cells, we evaluated the immunologic situation of patients in order to

Table 2. Demographics and clinical characteristics of CVID patients with allergic and without allergic disorders.

Clinical characteristics	Allergic CVID patients	Non-allergic CVID patients
Frequent middle ear infection	4	6
Respiratory infection	2	2
Lymphadenopathy	2	1
Lymphohyperplasia	2	0
Joint's pain	2	1
Bronchiectasis	1	3
Seizure	1	0
Brain encephalitis	1	0
Weight loss	1	0
Hyperplasia	1	0
Chronic diarrhea	0	1
Nephritic syndrome	0	1
Meningitis	0	1
Pneumonia	0	1
Malignant history	0	1
The onset age of clinical symptoms	2–20	2–10

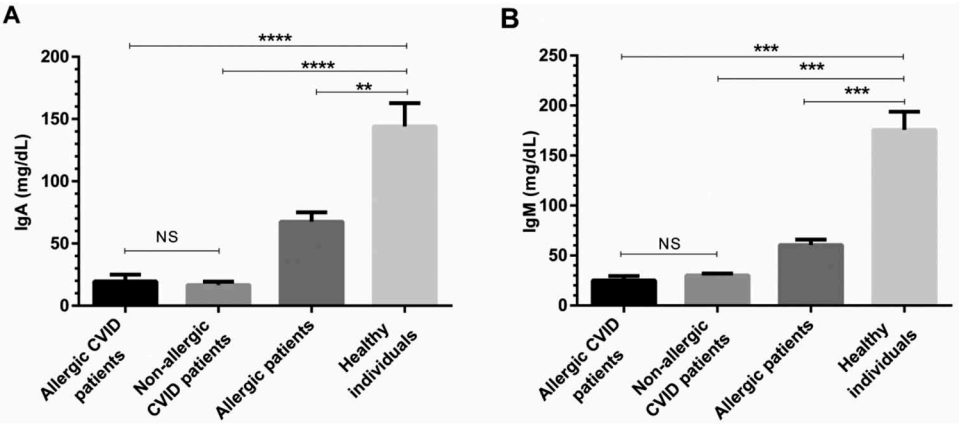

Figure 1. The serum concentrations of IgA and IgM in CVID patients, and control groups. The levels of IgA(A) and IgM (B) were measured by ELISA assay. The depicted results are representative of ten independent experiments for control groups and eight independent experiments for CVID patients. All data show mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$,**** $p < 0.0001$.

confirm CVID disease. The levels of IgM and IgA antibodies in the serum of allergic and non-allergic CVID patients were measured. A statistically significant reduction in the serum levels of IgA and IgM in allergic and CVID subjects with allergic and without allergic symptoms was observed compared to healthy controls, as expected ($p < 0.0001$–0.01, Figure 1A and B). However, no significant difference was observed in the levels of IgA and IgM between allergic and non-allergic CVID patients (Figure 1A and B).

Frequency of Tregs in healthy, CVID, allergic CVID, and allergic subjects

The percentage of Tregs in allergic, CVID patients, and healthy volunteers was determined. Our flow cytometry results showed that the percentage of Tregs was significantly decreased in both allergic and non-allergic CVID patients compared to healthy individuals and allergic patients ($p < 0.01$, Figure 2A and B). In addition, there was a significant difference in Treg percentage between allergic and healthy subjects ($p < 0.05$, Figure 2A and B). However, no statistically significant difference was observed in Treg percentage between CVID patients with allergic and without allergic symptoms (Figure 2A and B).

Expression level of p-stat5 in tregs in CVID, allergic CVID, allergic, and healthy subjects

Regarding known role of p-STAT5 in the development and survival of Tregs, the level of p-STAT5 in Tregs was measured. Our results indicated that there was a significant decrease in the level of p-STAT5 expression in Tregs in both allergic and non-allergic CVID patients compared to healthy subjects and allergic patients ($p < 0.0001$–0.001, Figure 3A–C). This significant reduction was also observed in allergic patients compared to healthy individuals ($p < 0.001$–0.05, Figure 3A-C). Although a numerical reduction in the average of p-STAT5 expression in allergic CVID patients was observed compared to non-allergic CVID, but this decrease was not statistically significant (Figure 3B and C).

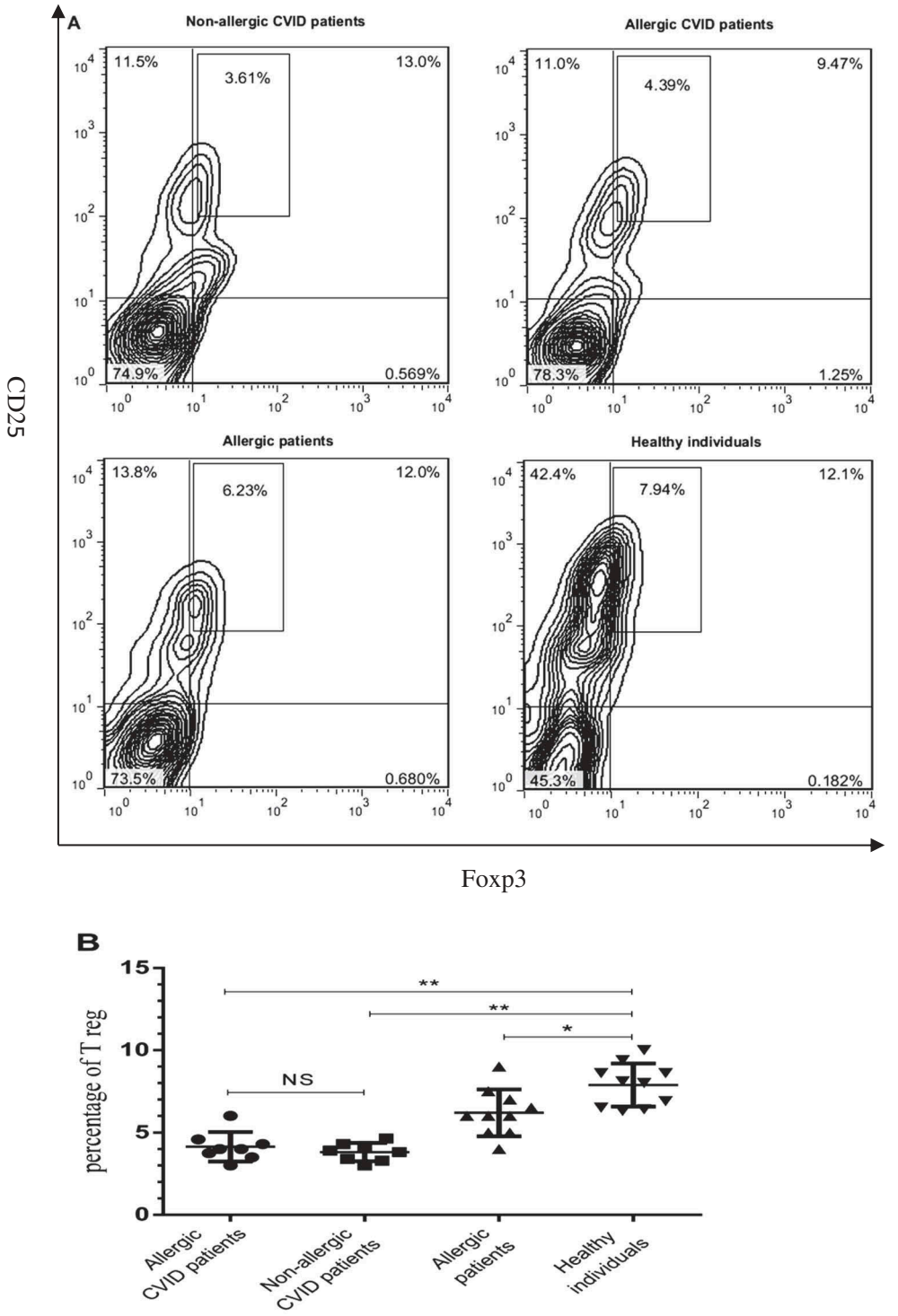

Figure 2. Percentage of Tregs in healthy volunteers, allergic, and CVID patients. PBMCs were isolated from healthy subjects, allergic and CVID patients and then stained with anti-human CD4, anti-human CD25, and anti-human Foxp3 antibodies. The percentage of Tregs was assessed using Flow cytometry (A) and then analyzed (B). The depicted results are representative of 10 independent experiments for control groups and eight independent experiments for patient groups. Data show mean ± SEM. *$p < 0.05$, **$p < 0.01$.

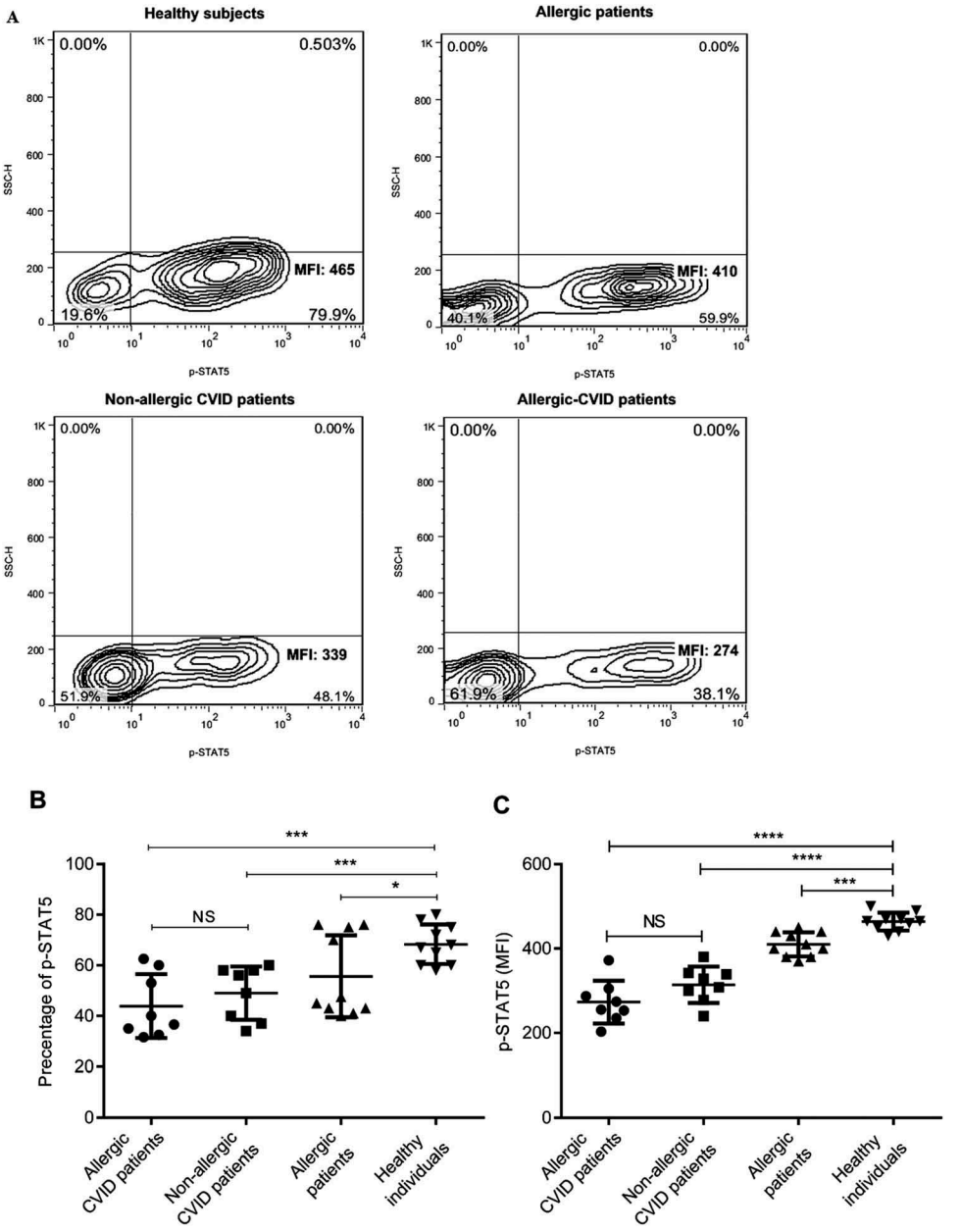

Figure 3. p-STAT5 expression in Tregs from healthy volunteers, allergic, and CVID patients. Tregs were isolated from PBMCs of healthy individuals, allergic, and CVID patients using an immunomagnetic separation method. Fixation and permeabilization of the cells were preformed for intracellular staining with PE anti-human p-STAT5 antibody. Data were acquired using Flow cytometry (A) and then analyzed (B and C). Data are representative of ten independent experiments for control groups and eight independent experiments for patient groups. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Discussion

CVID is a congenital disorder caused by inherent defects in the immune system that typically present with hypogammaglobulinemia, impaired antibody responses and recurrent bacterial infections (Arandi et al., 2013; Cunningham-Rundles and Bodian, 1999). CVID patients suffer from non-infectious complications such as cancer, autoimmunity, and splenomegaly (Aghamohammadi et al., 2006; Westh et al., 2017). Numerous studies have been conducted to address the causes of these complications in CVID subjects (Aghamohammadi et al., 2006; Azizi et al., 2016; Dadkhah et al., 2015). Some reports suggest that the reduction in the number and defects in the function of Tregs play a critical role in the pathogenesis of non-infectious complications in CVID patients (Arandi et al., 2013; Azizi et al., 2016). However, the possible role of Tregs in causing allergies in CVID patients is not fully identified so far. In the current study, we investigated whether the decreased number and impaired function of Tregs in CVID patients participate in susceptibility to allergy.

A growing body of evidence indicates that there is an increased tendency in the occurrence of allergic disorders in CVID patients (Agondi et al., 2010). Several mechanisms have been proposed to be responsible for developing allergy in CVID patients including: 1) frequent respiratory infections and high antibiotics usage; previous studies have shown that patients with hypogammaglobulinemia are more likely to have asthma and allergies due to frequent infections and high antibiotics usage (Dadkhah et al., 2015). Moreover, asthma can be concealed by repeated respiratory infections in CVID patients (Agondi et al., 2010). 2) Defect in IgA secretion; in patients with CVID and selective IgA deficiency, the reduced production of IgA causes mucosal immunodeficiency, which can facilitate the development of allergic reactions, inflammation, and bronchial asthma (Yazdani et al., 2016). 3) Th2 versus Th1 dominancy; several studies point to a bias of Th1 immunity toward Th2 responses in patients with CVID (Rezaei et al., 2008a; Rezaei et al., 2008b), although there are some papers that revealed a shift in immune responses toward Th1 phenotype (Cunill et al., 2017; Unger et al., 2017). 4) Impaired function and reduced number of Tregs; it has been reported that the defect in the function and reduction in the frequency of Tregs play a fundamental role in the pathogenesis of allergic disorders in CVID patients (Strickland and Holt, 2011). In this study, we unexpectedly observed that there was no significant difference in Treg percentage between allergic and non-allergic CVID patients, however, the proportion of Tregs within CD4+ subpopulation was significantly lower in the CVID patients then the control groups, which is consistent with other studies conducted on Tregs from patients with CVID (Bateman et al., 2012; Grace et al., 2009; Horn et al., 2009; Melo et al., 2009; Paquin-Proulx et al., 2013). Several lines of evidence demonstrated that the decreased number of Tregs participates in the pathogenesis of autoimmune diseases in CVID patients (Agondi et al., 2010; Dadkhah et al., 2015; Melo et al., 2009), while our data showed that the reduction in the number of Tregs did not have significant effects the incidence of allergic disorders in CVID subjects. Regarding that CVID disease is a heterogeneous disorder, it is likely that the development and exacerbation of allergic reactions in patients may be mediated by other unknown mechanisms and factors that some of them mentioned above. In line with this notion, Scheffold et al. showed that the development of allergic disorders may result in some Th2 cells escape from Treg control (Bacher et al., 2016).

Given that Tregs rely on p-STAT5 for their development, survival, and functions (Grace et al., 2009; Melo et al., 2009), the critical question was whether the reduction of Tregs in CVID patients was due to dysfunction of p-STAT5. The investigation of this protein expression revealed that p-STAT5 level significantly decreased in CVID patients compared to control groups, although no significant difference was observed in the level of p-STAT5 between CVID patients with allergic and without allergic symptoms. In agreement with these findings, Grace et al. indicated that the decreased level of p-STAT5 in CVID patients was significantly associated with the degree of T reg dysfunction (Grace et al., 2009). Moreover, in a clinical study conducted on CVID patients with autoimmune disease have demonstrated that p-STAT5b expression and Treg function was significantly decreased in patients with autoimmune diseases compared to patients without autoimmune diseases and healthy individuals (Nadeau et al., 2011). These results suggest that impairment of intracellular signaling in Tregs from CVID patients contribute to the reduction in the number of these cells. However, in contrast to other studies on CVID patients with autoimmune diseases, the results of this study indicated that the defect in the expression of p-STAT5 cannot affect susceptibility to allergic disorders in CVID patients through a reduction in the number of Tregs.

Taken together, the results of this study provide evidence to show that p-STAT5 signaling defect and decreased Treg number are not the main causes of allergy in CVID patients. Regarding the fact that allergy is a hypersensitivity of the immune system to innocuous environmental antigens, long-term use of antibiotics, environmental factors, Th2 versus Th1 dominancy, and weakness in mucosal immunity of CVID patients may be major causes that along with the reduced number and impaired function of Tregs make them more susceptible to allergic reactions than healthy individuals. However, more robust studies and studies with longitudinal design are required to clarify the role of environmental factors and Tregs in the pathogenesis of allergic disorders in CVID patients. Furthermore, it is noteworthy that the role of other molecules, which have a pivotal role in the development and survival of Tregs such as GITR (glucocorticoid-induced TNFR family related gene), Granzyme A, XCL1 (lymphotactin), and Foxp3 in causing allergies in patients is investigated.

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Conflict of interest

The authors report no conflicts of interest.

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