

## Evaluation of Regulatory B10 Cells in Common Variable Immunodeficiency Patients with and without Autoimmunity

Pouria Ghiaee<sup>1</sup>, Sarehsadat Ebrahimi<sup>2</sup>, Seyed Alireza Mahdavi<sup>3</sup>, Fatemeh Tarighat Monfared<sup>4</sup>, Marzieh Tavakol<sup>5</sup>, Mehrdad Dargahi Mal-Amir<sup>6</sup>, Nima Rezaei<sup>7,8</sup>, and Abdollah Jafarzadeh<sup>1</sup>

<sup>1</sup> Department of Immunology, School of Medicine, Kerman University of Medical Sciences, Kerman, Iran

<sup>2</sup> Department of Immunology and Allergy, Kerman University of Medical Sciences, Kerman, Iran

<sup>3</sup> Pediatric Respiratory Disease Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

<sup>4</sup> Pediatric Respiratory and Sleep Medicine Research Center, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran

<sup>5</sup> Non-Communicable Diseases Research Center, Alborz University of Medical Sciences, Karaj, Iran

<sup>6</sup> Air Pollution and Respiratory Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

<sup>7</sup> Research Center for Immunodeficiencies, Pediatrics Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran

<sup>8</sup> Primary Immunodeficiency Diseases Network (PIDNet), Universal Scientific Education and Research Network (USERN), Tehran, Iran

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### ABSTRACT

Common variable immunodeficiency disease (CVID) is the most prevalent symptomatic inborn errors of immunity, determined by defective B cell function, impaired antibody production, and susceptibility to frequent respiratory infections, enteropathy, autoimmunity, and malignancy. Due to the importance of autoimmunity in CVID and the probable role of regulatory B lymphocytes, we aimed to determine the frequency of B10 cells in CVID patients with and without autoimmunity.

A total of 24 CVID patients and 12 healthy controls were enrolled in the study. Patients were divided into two equal groups, with and without autoimmunity. Peripheral blood cells were stained with monoclonal antibodies (mAbs) to identify CD24<sup>hi</sup>CD38<sup>hi</sup> B cells, CD27<sup>int</sup> CD38<sup>+</sup> (plasmablasts), and CD24<sup>hi</sup>CD27<sup>+</sup> B cells by flow cytometry.

The percentages of B10, CD24<sup>hi</sup>CD27<sup>+</sup> and CD27<sup>int</sup> CD38<sup>+</sup> cells were significantly lower in total CVID patients, CVID patients with autoimmunity and CVID patients without autoimmunity compared to healthy controls (mean±standard deviation (SD) percentage of B10 cells: 6.36±9.21 (total CVID), 2.81±5.00 (CVID with autoimmunity), and 3.25±3.5 (CVID without autoimmunity) vs. 13.02±12.45 (healthy controls); CD24<sup>hi</sup>CD27<sup>+</sup> cells: 2.39±3.89, 3±5.20 and

**Corresponding Authors:** Abdollah Jafarzadeh, PhD;  
Department of Immunology, School of Medicine,  
Kerman University of Medical Sciences, Kerman, Iran. Tel: (+98  
34) 3132 0560, Fax: (+98 34) 3211 3170, Email:  
Jafarzadeh14@yahoo.com, jafarzadeh@kmu.ac.ir

Nima Rezaei, MD, PhD;  
Research Center for Immunodeficiencies, Pediatrics Center  
of Excellence, Children's Medical Center, Tehran University  
of Medical Science, Tehran, Iran. Tel: (+98 21) 6642 8998,  
Fax: (+98 21) 6692 3054, Email: rezaei\_nima@tums.ac.ir

1.78±1.94 vs. 20.38±14.27; CD27<sup>int</sup> CD38<sup>+</sup> cells: 6.80±18.49, 7.40±20.24 and 6.20±17.45 vs. 11.84±5.71). CVID patients without autoimmunity had a higher percentage of CD24<sup>hi</sup>CD38<sup>hi</sup> cells than CVID patients with autoimmunity (4.73±4.14 vs. 2.62±5.02).

The defect of regulatory B cells plays a significant role in the pathogenesis of autoimmunity in CVID. Further multicenter studies with higher sample sizes are suggested to determine the role of Breg cells in the clinical course of autoimmunity.

**Keywords:** Autoimmunity; CD24<sup>hi</sup>CD38<sup>hi</sup> B cells; Common variable immunodeficiency; Regulatory B cells

## INTRODUCTION

Inborn errors of immunity (IEI), previously known as primary immunodeficiencies (PID), are a heterogeneous group of congenitally disorders with defects in one or more components of the innate and/or adaptive immune system.<sup>1</sup> Common variable immunodeficiency (CVID) is the most frequent clinically significant IEI with an approximate prevalence of 0.5–6.9 per 100,000 individuals worldwide.<sup>2–5</sup> Despite the identification of numerous monogenic defects involved in various steps of maturation, activation, and survival of B cells as the underlying cause of CVID in about 20–50% of the patients, the exact pathogenesis of CVID in the majority of the patients is not well understood.<sup>6–8</sup> CVID is characterized by impaired antibody production and hypogammaglobulinemia, recurrent infectious episodes of the respiratory and gastrointestinal tracts, lymphoproliferation, granulomatous disease, malignancy, and autoinflammatory disorders.<sup>9</sup>

Autoimmunity is one of the most important causes of morbidity and mortality in CVID patients, with an overall prevalence of approximately 30%<sup>10–13</sup>. The most frequently observed autoimmune manifestations are autoimmune cytopenias (immune thrombocytopenia, autoimmune hemolytic anemia, autoimmune neutropenia, and pernicious anemia) followed by rheumatologic diseases (rheumatoid arthritis, Sjögren's syndrome, and systemic lupus erythematosus). Other, less common autoimmune phenomena including vitiligo, autoimmune thyroiditis, diabetes mellitus, multiple sclerosis, and alopecia.<sup>10–13</sup>

Dysregulated development of autoantibodies, increased number of circulating CD21<sup>low</sup> B lymphocytes. A reduced number of regulatory T cells with subsequently impaired suppressive function, defective regulation of apoptosis, and altered expression and signaling of toll-like receptors have been reported as

contributing factors to the pathogenesis of autoimmunity in CVID patients<sup>14–18</sup>.

Defects in the number or function of regulatory B lymphocytes, which exert a negative modulatory role on immune responses of cells could result in autoimmune and inflammatory diseases.<sup>19–23</sup> In humans, regulatory B lymphocytes that produce interleukin (IL)-10 are known as B10 cells and are further classified into IL-10-producing CD24<sup>hi</sup>CD38<sup>hi</sup>, CD24<sup>hi</sup>CD27<sup>+</sup>, and CD27<sup>int</sup> CD38<sup>+</sup> B cells.<sup>20,23–26</sup> Various studies have investigated the role of B10 cells in animal models of cancer and autoinflammatory diseases, attributing their effects to the production of IL-10 and tissue growth factor (TGF)- $\beta$ , and subsequently to the restoration of the TH1/TH2 balance, the induction of regulatory T cell expansion, and the inhibition of TH17 cells.<sup>27–29</sup> However, few studies have evaluated B10 cells and their role in CVID patients.<sup>30</sup>

A deeper understanding of the role of B10 cells in the immunopathogenesis of autoimmune phenomena in CVID could transform both diagnostic and therapeutic approaches for these patients. This could lead to earlier diagnosis and more effective management of CVID patients with autoimmune manifestations, thereby improving their quality of life and increasing their survival and overall well-being.

Given the significance of autoimmunity in CVID and the probable role of regulatory B lymphocytes in its pathogenesis, the current study aimed to compare the percentages of CD24<sup>hi</sup>CD38<sup>hi</sup>, CD24<sup>hi</sup>CD27<sup>+</sup>, and CD27<sup>int</sup>CD38<sup>+</sup> B cells, three essential subtypes of B10 cells between CVID patients with and without autoimmunity.

## MATERIALS AND METHODS

### Study Population

The current case-control study included 24 CVID patients both with and without autoimmunity, who were

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under clinical follow-up at the Children's Medical Center affiliated with Tehran University of Medical Sciences. Patients included in this study represent a typical CVID population with various symptoms and disease severity. The diagnosis of CVID was established based on the diagnostic criteria for IEI recommended by European Society for Immunodeficiencies (ESID). The criteria are as follows: Age at time of IEI diagnosis above 4 years old, profound decrease of IgG and of IgA with or without low IgM levels (defined as values 2 standard deviations below age-related norms on at least two separate occasions), at least one of the followings: increased susceptibility to infection, autoimmune manifestations, granulomatous disease, unexplained polyclonal lymphoproliferation and affected family member with antibody deficiency, along with poor antibody response to vaccines (and/or absent Isohemagglutinin) and/or low switched memory B cells (<70% of age-related average value). Exclusion criteria are secondary causes of hypogammaglobulinemia and no evidence of profound T-cell deficiency defined as percentage or absolute count of CD4<sup>+</sup> T cells below age-related average value and absence of T cell proliferation (<https://esid.org/Working-Parties/Registry-Working-Party/Diagnosis-criteria>). Patients with a secondary cause of immunodeficiency as well as CVID patients with monogenic defects identified through whole-exome sequencing (WES), were excluded from the study. The healthy controls (n=12) had no autoimmune manifestations, primary immunodeficiency disorders, and/or other immune system diseases. Patients and healthy individuals were matched by age and sex.

### Sample Size Formula

The sample size was estimated to be 24 patients based on the following sample size formula and a similar study.<sup>31</sup>

$$n = \frac{(Z_{1-\alpha/2} + Z_{1-\beta})^2 [P_1(1 - P_1) + P_2(1 - P_2)]}{(P_1 - P_2)^2}$$

$\alpha = 0.05$ ,  $Z_{1-\alpha/2} = 1.96$

$\beta = 0.02$ ,  $Z_{1-\beta} = 0.84$

$P_1 =$  B10 frequency in healthy subjects: 25.0% of Total B cells.

$P_2 =$  B10 frequency in CVID patients: 2.65% of Total B cells.

### Data Collection

Demographic, clinical, and immunologic data was collected from patients' medical records utilizing

a structured questionnaire. The collected data consisted of demographic data, age at onset of symptoms, age at the time of IEI diagnosis, delay which was defined as the interval between symptom onset and diagnosis, parental consanguinity, clinical manifestations, and laboratory data.

### Classification of the Patients

The patients were categorized into two groups, including patients with any autoimmune manifestations and those without. Patients were considered to have autoimmunity if any autoimmune manifestations, including immune thrombocytopenic purpura (ITP), pernicious anemia, autoimmune hemolytic anemia (AHA), celiac disease, atrophic gastritis, ulcerative colitis, systemic lupus erythematosus (SLE), psoriasis, juvenile rheumatoid arthritis, vitiligo, autoimmune hepatitis, inflammatory bowel disease (IBD) and alopecia were reported in their medical records. Autoimmunity was diagnosed by a specialist relevant to the affected organ or by an immunologist, based on established clinical and laboratory diagnostic criteria. Patients classified as non-autoimmune were those with no documented manifestations of autoimmunity in their medical records, no signs or symptoms indicative of autoimmune disease-including chronic inflammation, chronic fatigue, joint pain, and cutaneous lesions, and negative laboratory findings specific to autoimmune conditions.

### Sample Preparation and Flow Cytometry

Freshly collected whole blood samples were obtained and processed within 4 hours. To determine the proportions of B10 cell subsets in peripheral blood, immunophenotyping was performed using fluorochrome-conjugated anti-human murine monoclonal antibodies (mAbs). The following antibodies were utilized: anti-CD19-FITC (4G7), anti-CD24-PE (ML5), anti-CD38-PerCP (HB-7), and anti-CD27-allophycocyanin (APC) (M-T271). All antibodies were sourced from BioLegend (San Diego, USA). For surface staining, 100  $\mu$ L of whole blood was incubated with the antibodies for 20 minutes at 4°C in the dark. Following incubation, the stained cells were washed with phosphate-buffered saline (PBS) to remove any unbound antibodies. Subsequently, 1 mL of diluted FACS Lysing Solution was added and incubated for 10 minutes to lyse erythrocytes. Then, cells were centrifuged at 1500 RPM for five minutes, and the supernatant was discarded. The stained cells were rewashed with PBS, and all samples were kept cool and

protected from light until flow cytometry analysis. Flow cytometric acquisition of labeled B10 cell subsets was conducted on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA) to quantify the various B10 cell subpopulations.

### Identification of B10 Subsets by Flow Cytometry

Lymphocyte populations were initially identified by their forward scatter (FSC) and side scatter (SSC) properties. The total B cell population was defined by the expression of the lineage marker CD19. Within the CD19<sup>+</sup> B cell population, B10 subsets were specifically defined as follows: B10 cells were identified as CD19<sup>+</sup> CD24<sup>hi</sup> CD38<sup>hi</sup> (Transitional B cells), CD19<sup>+</sup> CD24<sup>hi</sup> CD27<sup>+</sup> (the human equivalent of B10 cells in mice), and CD19<sup>+</sup>CD27<sup>int</sup> CD38<sup>+</sup> (plasmablasts).

### t-SNE Analysis Reveals Distinct B Cell Clusters

To visualize multiparameter flow cytometry data, clustering and dimensionality reduction techniques are essential for automatically segregating cell populations while retaining the key features of each dataset. Currently, t-SNE is widely used in single-cell analysis. After assessing sample quality, B cells from individual samples were downsampled to 3,000 events per sample and then combined for further analysis.

### Statistical Analysis

Statistical analyses were performed using SPSS version 27 software (SPSS Inc., Chicago, IL, USA). Kolmogorov–Smirnov and Shapiro-Wilk tests were used to check the normality assumption. Central tendency and descriptive statistics were presented for quantitative data. For variables with skewed distributions, the mean±SD was reported as the index of data dispersion. Fisher’s exact test and Chi-square test were used to compare categorical variables, while independent-sample t-test, Mann–Whitney U test, and one-way analysis of variance (ANOVA) tests were applied to compare numerical variables. A *p* value<0.05 was considered statistically significant. Flow cytometry data were analyzed using FlowJo version 10.10 software. Graphs and charts were generated using GraphPad Prism version 10.0.2.

## RESULTS

### Study Population

The current study included 24 CVID patients and 12 healthy controls (male-to-female ratio, 1.4:1 and 1:1, respectively). Twelve patients were categorized as having autoimmune manifestations, and 12 were without. Detailed demographic data of CVID patients with and without autoimmunity as well as for healthy controls are shown in Table 1.

**Table 1. Demographic data of studied patients and healthy controls.**

Groups Parameters	CVID patients with autoimmunity	CVID patients without autoimmunity	Healthy controls	<i>P</i>
Sex (M/F)	6/6	8/4	6/6	0.900
Current age Mean ± SD (years)	33 ± 14.13	28 ± 19.63	30 ± 15.33	0.470
Parental consanguinity N (%)	7 (58)	6 (50)	-	0.680
Age at onset of symptoms Mean ± SD (years)	11 ± 11.66	11 ± 16.53	-	0.595
Age at the time of IEI diagnosis Mean ± SD (years)	23 ± 16.13	23 ± 15.5	-	0.774
Delay of diagnosis Mean ± SD (years)	8 ± 8.04	12 ± 10.02	-	0.321

CVID: common variable immunodeficiency; M: male; F: female; SD: standard deviation; N: number. \**p* value<0.05 is considered statistically significant.

### Clinical Manifestations

The frequency of autoimmune diseases observed among CVID patients with autoimmunity is depicted in Figure 1. AHA, rheumatoid arthritis (RA), and ITP were the most prevalent autoimmunities, each accounting for 25% of CVID patients with autoimmunity, followed by IBD (2 patients, 16.6%). Other autoimmune diseases were each observed only in one patient (8.3%). Detailed data regarding the clinical features of the studied patients are presented in Figure 2. Respiratory tract infections were the most common clinical manifestations among CVID patients, regardless of the presence or absence of autoimmunity. In descending order of frequency, the most common clinical manifestations among CVID patients with autoimmunity were sinusitis (75%), enteropathy (8.35%), chronic diarrhea (50%), and otitis media (50%). Among CVID patients without autoimmunity, the most frequent clinical manifestations were otitis media (50%), sinusitis (50%), pneumonia (50%), enteropathy (33.3%), and allergy (33.3%). Chronic diarrhea and arthritis were significantly more common among CVID patients with autoimmunity compared to CVID patients without autoimmunity (50% vs. 8%,  $p=0.027$  and 33% vs. 0%,  $p=0.037$ , respectively). No statistically significant differences were observed in other clinical features between the two groups (data not shown).

### Laboratory Findings

In the evaluation of immunological variables, the mean absolute count of CD3<sup>+</sup> T lymphocytes was significantly higher in CVID patients without autoimmunity compared to those with autoimmunity (3403.9 vs. 1578.7,  $p=0.03$ ). No significant differences were observed in other laboratory findings between the two groups. Table 2 represents detailed immunological data of studied patients.

### Flow Cytometry Analysis

For a more comprehensive assessment of potential in the percentage of regulatory B cells in CVID patients with and without autoimmunity, we compared the percentage of total lymphocytes, B lymphocytes, total B10 cells, CD24<sup>hi</sup>CD38<sup>hi</sup> cells, CD24<sup>hi</sup>CD27<sup>+</sup> cells, and CD27<sup>int</sup>CD38<sup>+</sup> in several groups including total CVID patients compared to healthy controls, CVID patients with autoimmunity compared to healthy controls, CVID patients without autoimmunity compared to healthy

controls and CVID patients with autoimmunity compared to CVID patients without autoimmunity (Table 3). Our analysis revealed no statistically significant differences in the percentages of total lymphocytes and B lymphocytes among the groups compared. However, the mean±SD percentages of total B10 cells, CD24<sup>hi</sup>CD27<sup>+</sup> cells, and CD27<sup>int</sup>CD38<sup>+</sup> were significantly lower in total CVID patients, CVID patients with autoimmunity, and CVID patients without autoimmunity in comparison to healthy controls. Mean±SD percentages of total B10 cells: 6.36±9.21, 2.81±5.00 and 3.25±3.5 versus 13.02±12.45 ( $p<0.001$ ). Mean±SD percentages of CD24<sup>hi</sup>CD27<sup>+</sup> cells: 2.39±3.89, 3±5.20 and 1.78±1.94 versus 20.38±14.27 ( $p<0.001$ ). Mean±SD percentages of CD27<sup>int</sup>CD38<sup>+</sup> B cells (plasmablasts): 6.80±18.49, 7.40±20.24 and 6.20±17.45 versus 11.84±5.71 ( $p<0.001$ ). CVID patients with autoimmunity had a lower mean±SD percentage of total B10 cells than CVID patients without autoimmunity. However, the difference was not statistically significant (2.81±5.00 vs. 3.25±3.50,  $p=0.108$ ). CD24<sup>hi</sup>CD38<sup>hi</sup> cells were significantly higher in healthy controls than total CVID patients and CVID patients with autoimmunity (mean ± SD percentage: 5.66±1.59 vs. 3.67±4.63,  $P$ -value=0.011 and 5.66±1.59 vs. 2.62±5.02,  $p=0.002$ ). Notably, CVID patients without autoimmunity had a statistically significantly higher percentage of CD24<sup>hi</sup>CD38<sup>hi</sup> cells than CVID patients with autoimmunity (mean±SD percentage: 4.73±4.14 vs. 2.62±5.02,  $p=0.039$ ). A comparison of total B10 cells, CD24<sup>hi</sup>CD38<sup>hi</sup>, CD24<sup>hi</sup>CD27<sup>+</sup>, and CD27<sup>int</sup>CD38<sup>+</sup> B cells between the groups described above is depicted in Figure 3.

To visualize multiparameter flow cytometric data, clustering and dimensionality reduction techniques are essential for automatically segregating cell populations while preserving key data features. Currently, t-SNE is the most widely used method in single-cell analysis. After assessing sample quality, B cells from each sample were down-sampled to 3000 events per sample and then combined for further study. The t-SNE results revealed distinct populations with phenotypes that matched those detected by flow cytometry using a four-color panel, indicating that our study's B cell staining protocols were stable and reliable (Figure 4).

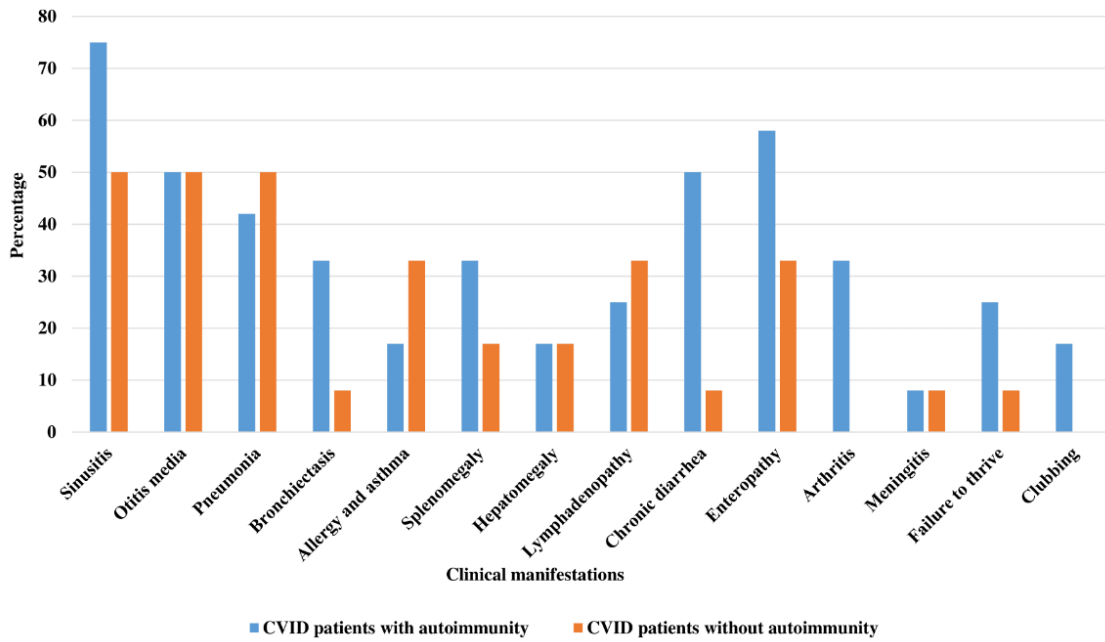


Figure 1. The frequency of autoimmune diseases in CVID patients with autoimmunity.

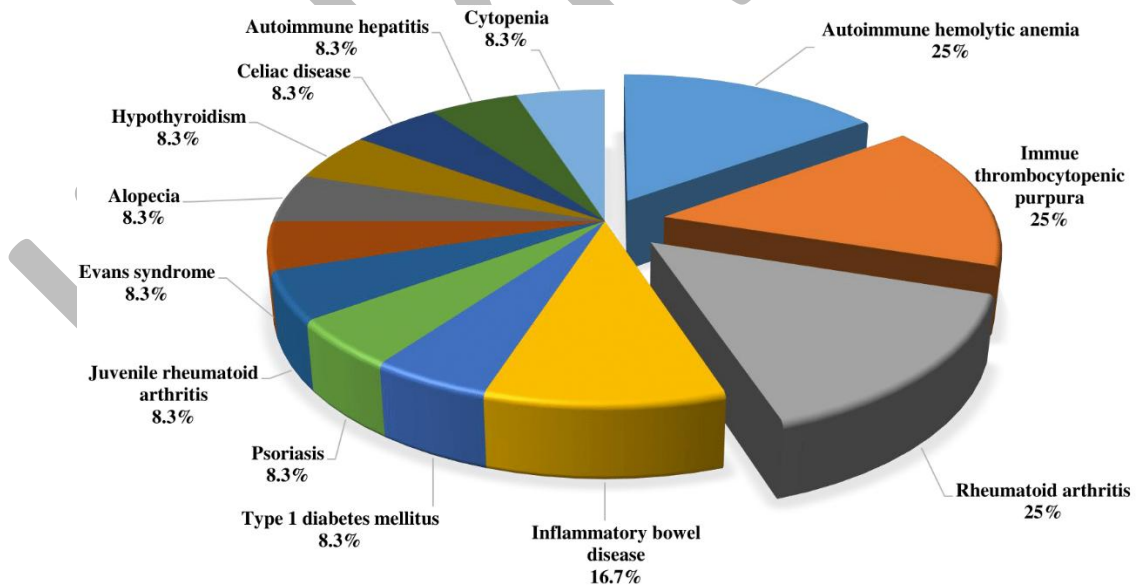


Figure 2. The clinical manifestations of studied CVID patients with and without autoimmunity.

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**Table 2. Immunologic data of studied patients.**

Parameters	Groups	CVID patients with autoimmunity	CVID patients without autoimmunity	<i>P</i>
WBC		9 (4-14.5)	10 (4-13)	0.43
Mean (min, max), *10 <sup>3</sup> cells/μL				
Lymphocytes		2652.3 (884.4-5562.6)	3959.4 (457-16151)	0.77
Mean (min, max), cells/μL				
Neutrophils		4195.8 (23.3-9198)	5971.2 (2016-19685)	0.48
Mean (min, max), cells/μL				
Platelets		239.5 (25-424)	238 (9-621)	0.46
Mean (min, max), *10 <sup>3</sup> cells/μL				
CD3 <sup>+</sup> cells		1578.7 (42.3-3518.6)	3403.9 (297-11305.7)	0.03*
Mean (min, max), cells/μL				
CD4 <sup>+</sup> cells		849.5 (221.1-1607.6)	1292.4 (68.5-4009)	0.25
Mean (min, max), cells/μL				
CD8 <sup>+</sup> cells		1025.2 (406.4-2280.7)	2069.8 (228.5-8398.5)	0.48
Mean (min, max), cells/μL				
CD19 <sup>+</sup> cells		153.7 (26.5-278.1)	341.8 (0-1313.1)	0.45
Mean (min, max), cells/μL				
CD20 <sup>+</sup> cells		206.2 (201.1-211.4)	282.6 (123.4-441.8)	0.51
Mean (min, max), cells/μL				
CD16 <sup>+</sup> cells		193.6 (47.6-418.9)	392.8 (0-1300.2)	0.08
Mean (min, max), cells/μL				
CD56 <sup>+</sup> cells		131.6 (0-293.2)	339.2 (164.5-650.1)	0.35
Mean (min, max), cells/μL				
IgG		192 (0-450)	392 (41-771)	0.47
Mean (min, max), mg/dL				
IgM		41 (4-134)	34.35 (2-178.5)	0.43
Mean (min, max), mg/dL				
IgA		12 (0-34)	27 (1-95)	0.50
Mean (min, max), mg/dL				
IgE		1 (1-2)	110 (0.1-481)	0.69
Mean (min, max), IU/mL				

CVID: common variable immunodeficiency; WBC: white blood cells; μL: microliter; CD: cluster of differentiation; Ig: immunoglobulin; mg: milligram; dL: deciliter; IU: international units; mL: milliliter.

\**p* value<0.05 is considered statistically significant.

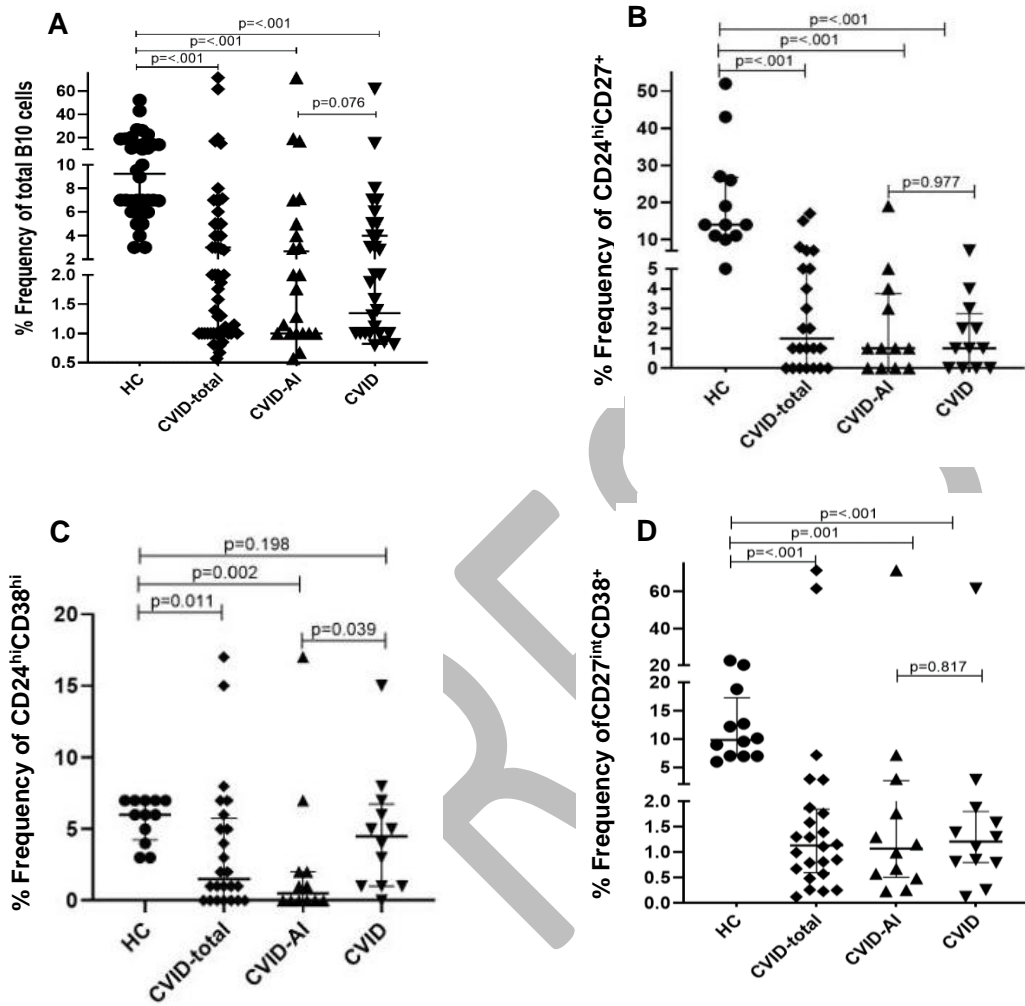
**Table 3. Comparison of lymphocytes, B cells, total B10 cells, their subclasses, and plasmablasts between healthy controls and CVID patients with and without autoimmunity.**

Groups	Lymphocytes Mean±SD	<i>p</i>	B cells Mean ± SD	<i>p</i>	Total B10 cells Mean±SD	<i>p</i>	CD24 <sup>hi</sup> CD27 <sup>+</sup> cells Mean ± SD	<i>p</i>	CD24 <sup>hi</sup> CD38 <sup>hi</sup> cells Mean±SD	<i>p</i>	CD27 <sup>int</sup> CD38 <sup>+</sup> cells Mean±SD	<i>p</i>
<b>Total CVID patients (N=24)</b>	32.44±14.3 8		9.36±8.58		6.36±9.21		2.39 ± 3.89		3.67 ± 4.63		6.80 ±18.49	
<b>Healthy controls (N=12)</b>	29.91±9.07	0.631	8.91±3.26	0.655	13.02±12.45	<0.001*	20.38 ± 14.27	<0.001*	5.66 ± 1.59	0.011*	11.84 ±5.71	<0.001*
<b>CVID patients with autoimmunity (N=12)</b>	31.48±13.5 8		9.22±10.00		2.81±5.00		3 ± 5.20		2.62 ± 5.02		7.40 ±20.24	
<b>Healthy controls (N=12)</b>	29.91±9.07	0.755	8.91±3.26	0.551	13.02 ± 12.45	<0.001*	20.38 ± 14.27	<0.001*	5.66 ± 1.59	0.002*	11.84 ±5.71	0.001*
<b>CVID patients without autoimmunity (N=12)</b>	33.41±15.6 8		9.50±7.33		3.25 ± 3.5		1.78 ± 1.94		4.73 ± 4.14		6.20 ±17.45)	
<b>Healthy controls (N=12)</b>	29.9 ±9.07	0.630	8.91±3.26	0.887	13.02 ± 12.45	<0.001*	20.38 ± 14.27	<0.001*	5.66 ± 1.59	0.198	11.84±5.71	<0.001*
<b>CVID patients with autoimmunity (N=12)</b>	31.48±13.5 8		9.22±10.00		2.81 ± 5.00		3 ± 5.20		2.62 ± 5.02		7.40 ±20.24	
<b>CVID patients without autoimmunity (N=12)</b>	33.41±15.6 8	0.799	9.50±7.33	0.755	3.25 ± 3.50	0.108	1.78 ± 1.94	0.977	4.73 ± 4.14	0.039*	6.20 ±17.45	0.817

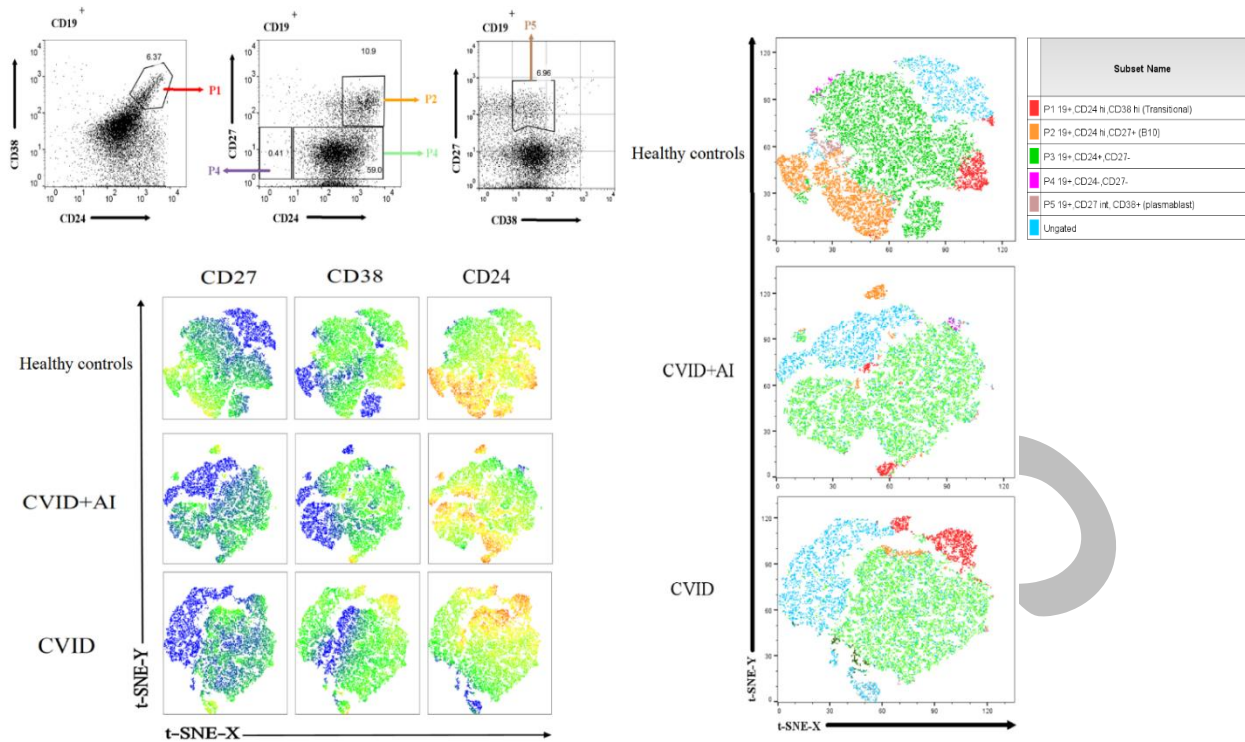
SD: standard deviation; N: number;

\**p*<0.05 is considered statistically significant

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**Figure 3.** Comparison of total B10 cells (A), CD24<sup>hi</sup>CD27<sup>+</sup> (B) CD24<sup>hi</sup>CD38<sup>hi</sup> (C) CD27<sup>int</sup>CD38<sup>+</sup> (D) B cells between healthy controls (HC), total CVID patients (CVID-total), CVID patient with autoimmunity (CVID-AI) and CVID patients without autoimmunity (CVID).



**Figure 4. t-SNE analysis objectively delineates B cell subsets in the peripheral blood of healthy individuals and patients. t-SNE-based dimension reduction was performed on B cells, revealing distinct, shared, and diverse lymphocyte phenotypes (Figure 4). Transitional B cells: CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> B cells; B10 cells: CD19<sup>+</sup>CD24<sup>hi</sup>CD27<sup>+</sup> B cells; plasmablasts: CD19<sup>+</sup>CD27<sup>int</sup>CD38<sup>+</sup> B cells**

## DISCUSSION

Although regulatory B10 cells may contribute to autoinflammation, their role on CVID patients remains incompletely understood. In the current case-control study, we evaluated the possible involvement of these cells in the mechanism of autoimmunity in CVID patients by comparing the percentage of B10 cells and their subsets, CD24<sup>hi</sup>CD27<sup>+</sup>, CD24<sup>hi</sup>CD38<sup>hi</sup>, and CD27<sup>int</sup>CD38<sup>+</sup> B cells between CVID patients and healthy controls as well as CVID patients with and without autoimmunity. B10 cells and their subsets were significantly lower in CVID patients than in healthy controls; however, a comparison of these cells between CVID patients with autoimmunity and CVID patients without autoimmunity demonstrated that only CD24<sup>hi</sup>CD38<sup>hi</sup> cells were remarkably lower in CVID patients with autoimmune manifestations, suggesting the possible role for these cells in the pathogenesis of autoimmunity in CVID. According to our findings, there was no significant difference in the percentage of total lymphocytes and B lymphocytes between CVID patients

and healthy controls. However, regulatory B10 cells and their subtypes, CD24<sup>hi</sup>CD27<sup>+</sup> and CD24<sup>hi</sup>CD38<sup>hi</sup> cells, were remarkably decreased in CVID patients compared with healthy controls, regardless of autoimmunity. This finding aligns with several previous studies,<sup>30,32</sup> suggesting that the underlying pathogenesis in CVID mainly compromises the terminal differentiation of B cells to plasma cells and memory B cells, leading to diminished levels of switched memory B cells, marginal zone B cells, regulatory B cells, and plasmablasts as the primary B cell defects observed among CVID patients.<sup>32-34</sup> Of interest, a minority of CVID patients may have diminished peripheral B cell counts, which can be attributed to arrest in early B cell differentiation steps in the bone marrow, impaired B cell survival factors, the presence of negative B cell regulators in the periphery, or increased B cell apoptosis.<sup>34-36</sup>

Based on our findings, the percentage of CD24<sup>hi</sup>CD38<sup>hi</sup> cells was significantly different between patients with and without autoimmunity. CD24<sup>hi</sup>CD38<sup>hi</sup> cells were decreased in the group with autoimmunity compared with those without autoimmunity.

## Breg Cells in CVID with and without Autoimmunity

Several studies have investigated the role of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> cells in autoimmunity [37-40]. Wang et al reported that CD24<sup>hi</sup>CD38<sup>hi</sup> B cells were decreased in the peripheral blood of patients with type 1 diabetes (T1D) compared with healthy controls ( $5.6 \pm 3.5$  versus  $6.9 \pm 3.3\%$ ).<sup>40</sup> Moreover, in another study, the number of CD24<sup>hi</sup>CD38<sup>hi</sup> B cells was reduced in patients with RA compared to healthy individuals.<sup>41</sup> The results of these studies are consistent with the current findings. These findings suggest that different B10 cell subsets may affect autoimmune manifestations in CVID patients differently. CD24<sup>hi</sup>CD38<sup>hi</sup> cells are essential in regulating the immune system and preventing excessive inflammatory responses. Under normal conditions, these cells help control inflammation and avoid autoimmunity by producing anti-inflammatory cytokines such as IL-10. A decrease in CD24<sup>hi</sup>CD38<sup>hi</sup> B cells in CVID patients with autoimmunity may impair the immune system's ability to control inflammation.<sup>30,42</sup>

Contrary to our findings, Zhu et al reported that the percentage of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> cells was remarkably increased in pemphigus patients compared to healthy individuals [43]. The exact role of CD24<sup>hi</sup>CD38<sup>hi</sup> B cells in autoimmune patients and their correlation to the maintenance of peripheral tolerance remains obscure.<sup>37</sup> According to our findings, CD24<sup>hi</sup>CD27<sup>+</sup> cells were significantly higher in healthy controls than in total CVID patients and CVID patients with autoimmunity. In line with our results, Barsotti et al reported that CVID patients had lower levels of CD24<sup>hi</sup>CD27<sup>+</sup> compared to healthy controls;<sup>30</sup> however, Daïen et al reported that frequencies of CD24<sup>hi</sup>CD27<sup>+</sup> B cells were similar in patients with RA and healthy controls.<sup>25</sup> Current results suggest that CVID patients may have defects in the regulatory B cells described as the main source of IL-10 in humans due to a lower frequency of the CD24<sup>hi</sup>CD27<sup>+</sup> cells subpopulation. In our study, plasmablasts (CD27<sup>int</sup>CD38<sup>+</sup> cells) were significantly lower in total CVID patients and CVID patients with and without autoimmunity in comparison to healthy controls, which can be attributed to the defective terminal differentiation of B cells to plasma cells and memory B cells in CVID.<sup>32-34</sup> However, we found no difference in the percentage of these cells between CVID patients with autoimmunity and those without it. On the contrary, CD27<sup>int</sup>CD38<sup>+</sup> cells have been reported as a specific subtype IL-10-producing regulatory B cell subtype that suppresses autoimmunity through IL-10 secretion [44]. In this regard, mice

lacking plasmablasts developed an exacerbated experimental autoimmune encephalomyelitis (EAE) <sup>44</sup>. To the best of our knowledge, no studies have evaluated the role of CD27<sup>int</sup>CD38<sup>+</sup> cells in the pathogenesis of autoimmunity in CVID. Further studies are required to assess the potential role of CD27<sup>int</sup>CD38<sup>+</sup> cells in autoimmune manifestations in CVID patients, which could provide a basis for exploring new therapeutic strategies.

The influential role of regulatory B cells in patients with autoimmunity is supported by the finding that the percentage of CD24<sup>hi</sup>CD38<sup>hi</sup> cells is altered in these patients. By confirming our results, Breg-based therapies may be able to modify the disease course of autoimmune disorders. Further multicenter studies with higher sample sizes are suggested to determine the role of Breg cells in the clinical course of autoimmunity.

### STATEMENT OF ETHICS

This study was approved by the Ethics Committee of the Kerman University of Medical Sciences (IR.KMU.AH.REC.1402.186), and written informed consents were obtained from all patients and/or their legal guardians.

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### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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### DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

### AI ASSISTANCE DISCLOSURE

Not applicable.

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