ORIGINAL ARTICLE

Circulating Helper T-Cell Subsets and Regulatory T Cells in Patients With Common Variable Immunodeficiency Without Known Monogenic Disease

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Abstract

Background: Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (PID). It is characterized by heterogeneous clinical manifestations and defects in B cells and T cells. In the present study, we investigated helper T (T_H) cell subsets and regulatory T (Treg) cells and their related cytokines and transcription factors in CVID patients with no definitive genetic diagnosis.
Methods: The study population comprised 13 CVID patients and 13 healthy controls. Mutation analysis was performed using whole exome sequencing in CVID patients to rule out monogenic PIDs. T_H subsets and Treg were analyzed using flow cytometry. The expression of determinant cytokines (IFN-γ, IL-17, IL-22, and IL-10) and cell subset specific transcription factors was evaluated before and after stimulation.
Results: The main clinical presentations of these patients were infections only and lymphoproliferative phenotypes. No autoimmune or allergy phenotypes were recorded. The frequencies of $CD4^+$ T cells, T_H17, and Treg cells were significantly reduced in CVID patients; however, T_H1, T_H1-like T_H17, and T_H22 subsets were normal. After stimulation, expression of retinoic-acid-orphan-receptor-C (*RORC*), runt-related transcription factor 1 (*RUNX1*), *IL17*, and IL10 was significantly lower in CVID patients than in the healthy controls. Moreover, the concentration of IL-17 and IL-10 in the cell culture supernatants of stimulated $CD4^+$ T cells was lower in CVID patients than in healthy controls.
Conclusions: Our findings demonstrate that the imbalance of T_H17 and Tregs could be associated with infection and the lymphoproliferative phenotype in CVID patients without monogenic disorders.

Key words: Common variable immunodeficiency. CVID. Infection. Autoimmunity. Lymphoproliferative disorder. Regulatory T cell. Helper T cell.

Resumen

Antecedentes: La inmunodeficiencia variable común (CVID) es la inmunodeficiencia primaria (PID) sintomática más frecuente, caracterizada por manifestaciones clínicas heterogéneas y alteraciones de los linfocitos B y T. En este trabajo, investigamos las poblaciones de linfocitos T cooperadores (Th) y linfocitos T reguladores (Treg), así como sus citocinas y factores de transcripción, en pacientes con CVID sin un diagnóstico genético definitivo.
Métodos: Se estudiaron 13 pacientes con CVID y 13 controles sanos (HC). El análisis de las mutaciones se realizó mediante secuenciación del exoma completo en los pacientes con CVID para descartar PID monogénicas. Las poblaciones de linfocitos Th y Treg se examinaron mediante citometría de flujo. Se cuantificaron las citocinas características (IFN-γ, IL-17, IL-22 e IL-10) y los factores de transcripción específicos de estas subpoblaciones linfocitarias, tanto antes como después de la estimulación.
Resultados: Las principales manifestaciones clínicas de estos pacientes fueron las infecciones y los fenotipos linfoproliferativos, pero no se encontraron fenotipos autoinmunes ni de enfermedad alérgica. Los porcentajes de linfocitos T CD4+, Th17 y linfocitos Treg se

redujo significativamente en los pacientes con CVID; sin embargo, las poblaciones de Th1, Th1similares a Th17 y Th 22 fueron normales. Después de la estimulación, la expresión de los genes receptor huérfano tipo C del ácido retinoico (RORC) y del factor de transcripción 1 relacionado con Runt (RUNX1), IL-17 e IL-10 fue significativamente menor en los pacientes con IDCV en comparación con los controles sanos. También se objetivó una menor concentración de IL-17 e IL-10 en los sobrenadantes del cultivo de linfocitos T CD4 + estimulados de los pacientes con CVID, respecto a los HC.
Conclusiones: Nuestros hallazgos demuestran que en los pacientes con CVID sin un diagnóstico genético definitivo y sin trastornos monogénicos, el desequilibrio de Th17 y Treg podría estar asociado con infecciones y fenotipos linfoproliferativos.

Palabras clave: Inmunodeficiencia variable común. CVID. Infección. Autoinmunidad. Linfoproliferación. Linfocito T regulador. Linfocito T cooperador.

Introduction

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (PID). It is characterized by heterogeneous clinical/immunologic manifestations [1], including susceptibility to recurrent infections, hypogammaglobulinemia, and reduced specific antibody response to protein and polysaccharide antigens [2]. The underlying causes of CVID are largely unknown. Genetic mutations can be identified as the cause of disease in approximately 10%-20% of patients depending on the ethnicity and population structure of the cohort [3]. The genes involved include mainly the members of the B-cell coreceptor complex, transmembrane activator and calcium modulator and cyclophilin ligand interactor (*TACI*), B cell–activating factor receptor (*BAFFR*), inducible costimulator (*ICOS*), and lipopolysaccharide responsive beige-like anchor (*LRBA*) genes. Although these new monogenic defects share clinical phenotypes with CVID, they could be considered distinct PIDs with a CVID-like phenotype [3-5]. However, a definite molecular genetic diagnosis has not been made in more than 80% of clinically diagnosed CVID patients, and the cause of their disease remains unknown.

In addition to recurrent infections, CVID patients have a wide range of clinical manifestations, including autoimmune disease, allergic diseases, enteropathy, and lymphoproliferative disorders [6-8]. Several studies reported that except for severe B-cell deficiency, T-cell abnormalities may also be involved in the pathogenesis of immune dysregulation in CVID patients. The T-cell defects that characterize CVID include $CD4^+$ T-cell deficiency, defects in regulatory T cell (Treg) counts and function, decreased lymphocyte proliferation, uncontrolled T-cell polarization, elevated levels of T-cell activation markers, and abnormal cytokine production [9-13].

Although the abnormality of $CD4^+$ T cells has been linked to clinical presentations of CVID including lymphoproliferative and autoimmune disorders [11], no studies have examined the frequencies and functional status of T_H subsets and their intricate balance with Treg cells. In the present study, we evaluated the proportion and frequencies of peripheral T_H1, T_H1-like T_H17, T_H17, T_H22, and $CD127^{low/-}$ Treg cells, as well as their determinant cytokines and transcriptional factors in CVID patients with no definitive genetic diagnosis.

Patients and Methods

Patients

The study population comprised available CVID patients who were referred to the PID Clinic at the Children's Medical Center affiliated to Tehran University of Medical Sciences, Tehran, Iran. Thirteen healthy individuals with no history of immune disorders (eg, severe infection, allergy, autoimmunity, or malignancy) were selected as a healthy control (HC) group. The study was approved by the Ethics Committee of Tehran University of Medical Sciences (ID number: IR.TUMS.REC.1394.696), and written informed consent was obtained from all participants and/or their parents. Demographic and clinical data were collected from the Iranian national registry of PID patients [14], which was updated at monthly visits and thoroughly reviewed by clinical immunologists. The inclusion criteria were a definitive diagnosis of CVID, as defined by the European Society for Immunodeficiencies (https://esid.org/Education/Diagnostic-Criteria-PID) [2], and regular therapy with intravenous Ig (IVIG).

Whole Exome Sequencing

Genomic DNA was extracted from whole blood from each proband, randomly fragmented, amplified by ligation-mediated polymerase chain reaction (PCR), and captured and sequenced according to the manufacturer's protocol, as described previously [15]. After raw image file processing, sequences were generated and aligned to the human genome reference (UCSC hg 19 version; build 37.1). We followed the analysis protocol for whole exome sequencing designed by the BGI-Shenzhen/Karolinska Institute as described previously [16]. Patients with a tentative diagnosis of CVID and an identified mutation within 373 known monogenic PID genes (Table S1) were excluded from this study [15,16]. Therefore, the study population comprised 13 CVID patients with no definitive genetic diagnosis.

Cell Isolation and Purification of $CD4^+$ T Cells

The blood samples were collected 4 weeks after the IVIG infusion in heparin-containing tubes. Lymphocyte counts were performed using the Sysmex KX-21N Hematology Analyzer

(Sysmex Corporation). Peripheral blood mononuclear cells (PBMCs) were obtained from both patients and HCs using lymphocyte separation medium (Lymphosep, Biosera) and resuspended in RPMI medium (Lymphosep, Biosera) supplemented with 10% fetal bovine serum (Lymphosep, Biosera), penicillin (100 IU), and streptomycin (100 µg/mL) (Biosera, Ringmer) for immunophenotyping and $CD4^+$ T-cell isolation. The viability of isolated PBMCs was more than 97%, as assessed using the Trypan blue viability test. $CD4^+$ T cells were purified from PBMCs with a human $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec) by depletion of non–$CD4^+$ T cells (negative selection). The purity of $CD4^+$ T cells was routinely more than 95% based on flow cytometry.

Flow Cytometry Analysis

For intracellular staining of T_H cells, 1×10^6 PBMCs were stimulated with phorbol myristate acetate (PMA, 50 ng/mL, Sigma) and ionomycin (1 µg/mL, Sigma) for 5 hours in RPMI medium at 37°C in a 5% CO_2 humidified atmosphere in the presence of Brefeldin A (BFA, 10 µg/mL, eBioscience). The stimulated cells were washed with cold PBS, and cell surface staining antibodies (anti-CD4 PerCP-cy5.5, clone OKT4) (eBioscience) were added and incubated in the dark at 4°C for 30 minutes. Alternatively, for evaluation of Tregs, unstimulated PBMCs were stained using surface antibodies (anti-CD4 PerCP-cy5.5, anti-CD25 APC [clone BC96], and anti-CD127 FITC [clone eBioRDR5]) (eBioscience) and were incubated in the dark at 4°C for 30 minutes. Following surface staining, the cells were washed twice, fixed and permeabilized with Fix/Perm buffer, and suspended in permeabilization buffer (eBioscience). Intracellular cytokine staining antibodies (anti-IFN-γ FITC [clone CZ-4], anti-IL-17 PE [clone eBio64DEC17], and anti-IL-22 APC [clone IL22JOP] for evaluation of T_H subsets and anti-FoxP3 PE [Forkhead Box P3, clone 236A/E7] for evaluation of Treg) were added and incubated at room temperature for 30 minutes. Cells were washed with permeabilization buffer, resuspended in cold staining buffer, and counts were determined using a BD FACSCalibur Flow Cytometer (BD Biosciences). Lymphocytes were gated on forward and side scatter profiles and analyzed using FlowJo software (Tree Star). The percentage of T_H1, T_H1-like T_H17, T_H17, and T_H22 lymphocytes was acquired by calculating, respectively, the percentage of IFN-γ^+IL-17^-, IFN-γ^+IL-17^+, IFN-γ^-IL-17^+, and IFN-γ^-IL-17^-IL-22^+ cells within a $CD4^+$ population. The percentage of Treg lymphocytes was acquired by calculating the percentage of $CD25^+FoxP3^+$ cells within a $CD4^+$ population. CD127 expression was also evaluated on $CD4^+CD25^+FoxP3^+$ cells. Isotype-matched control antibodies and fluorescence minus one (FMO) control stains were used to determine background levels of staining.

Quantitative Real-time Polymerase Chain Reaction (RT-PCR)

$CD4^+$ T cells were harvested and brought to a final concentration of 2×10^6/mL in 24-well plates that were precoated with 3 µg/mL anti-CD3 mAb. Moreover, 2 µg/mL anti-CD28 mAb was added concurrently and incubated in RPMI medium at 37°C in a 5% CO_2 humidified atmosphere. Stimulated cells were collected after 18 hours and washed twice with PBS. For RT-PCR, total RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen) and reversed transcribed into cDNA using the Takara kit (Takara) according to the manufacturer's instructions with some modifications. Expression levels of *IFNG, IL17, IL22, IL10*, T-box transcription factor (*TBET*), runt-related transcription factor 1 (*RUNX1*), RAR-related orphan receptor C (*RORC*), aryl hydrocarbon receptor (*AHR*), and *FOXP3* genes were measured with quantitative RT-PCR using SYBR Green PCR Master Mix (Takara) with specific primers (Table S2). Quantitative gene expression data were normalized relative to levels of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase).

Cytokine Assay

$CD4^+$ T cells were harvested and brought to a final concentration of 3×10^5/mL in 24-well plates before being stimulated with anti-CD3 mAb and anti-CD28 mAb using the method mentioned in the previous section. Unstimulated cells were used as controls for each experiment. Supernatants were collected after 48 hours, and cytokine production (IFN-γ, IL-17, IL-22, and IL-10) was evaluated using quantitative enzyme-linked immunosorbent assay (ELISA) with the commercial human ELISA Ready-SET-Go kits (eBioscience) according to the manufacturer's instructions. The sensitivity of detection was 2 pg/mL for IL-10, 4 pg/mL for IFN-γ and IL-17, and 8 pg/mL for IL-22.

Statistical Analysis

Values were expressed as frequency (number and percentage) and median (IQR), as appropriate. The Shapiro-Wilks test was used to check normality. A parametric or nonparametric test was

Table. Demographic Data and Clinical Characteristics of CVID Patients and HCs

Parameters	CVID (N=13)	HC (N=13)
Male/female	6/7	6/7
Consanguinity, No. (%)	8 (61.6)	4 (30.8)
Median (IQR) age at the time of the study, y	14.0 (10.0-29.0)	14.0 (10.0-29.0)
Median (IQR) age at onset of symptoms, y	4.0 (0.75-7.0)	–
Median (IQR) age at the time of diagnosis, y	9.0 (7.0-24.0)	–
Median (IQR) delay in diagnosis, y	9.0 (7.0-24.0)	–
Infection only phenotype, No. (%)	5 (38.5)	–
Autoimmunity, No. (%)	0 (0.0)	–
Enteropathy, No. (%)	1 (7.7)	–
Lymphoproliferative disorder, No. (%)	7 (53.8)	–
Allergy, No. (%)	0 (0.0)	–

Abbreviations: CVID, common variable immune deficiency; HC, healthy control.

then applied. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22 (IBM Corp).

Results

Characteristics and Clinical Phenotypes of CVID Patients

To determine the frequency of different subsets of $CD4^+$ T cells, a total of 13 Iranian CVID patients (6 males and 7 females) without a genetic diagnosis after whole exome sequencing were compared with 13 sex-age matched HCs. The demographic and clinical characteristics of patients are summarized in the Table. The first presentation for immunodeficiency was infection in 11 (84.6%) patients and chronic diarrhea in 2 (15.4%). In total, 10 CVID patients (77%) had a history of respiratory tract infections (RTIs), and 4 (30.8%) had skin infections. Lymphoproliferative disorders were observed in 7 (53.8%) patients. Bronchiectasis, arteritis, and failure to thrive were observed in 2 patients each (15.4%),

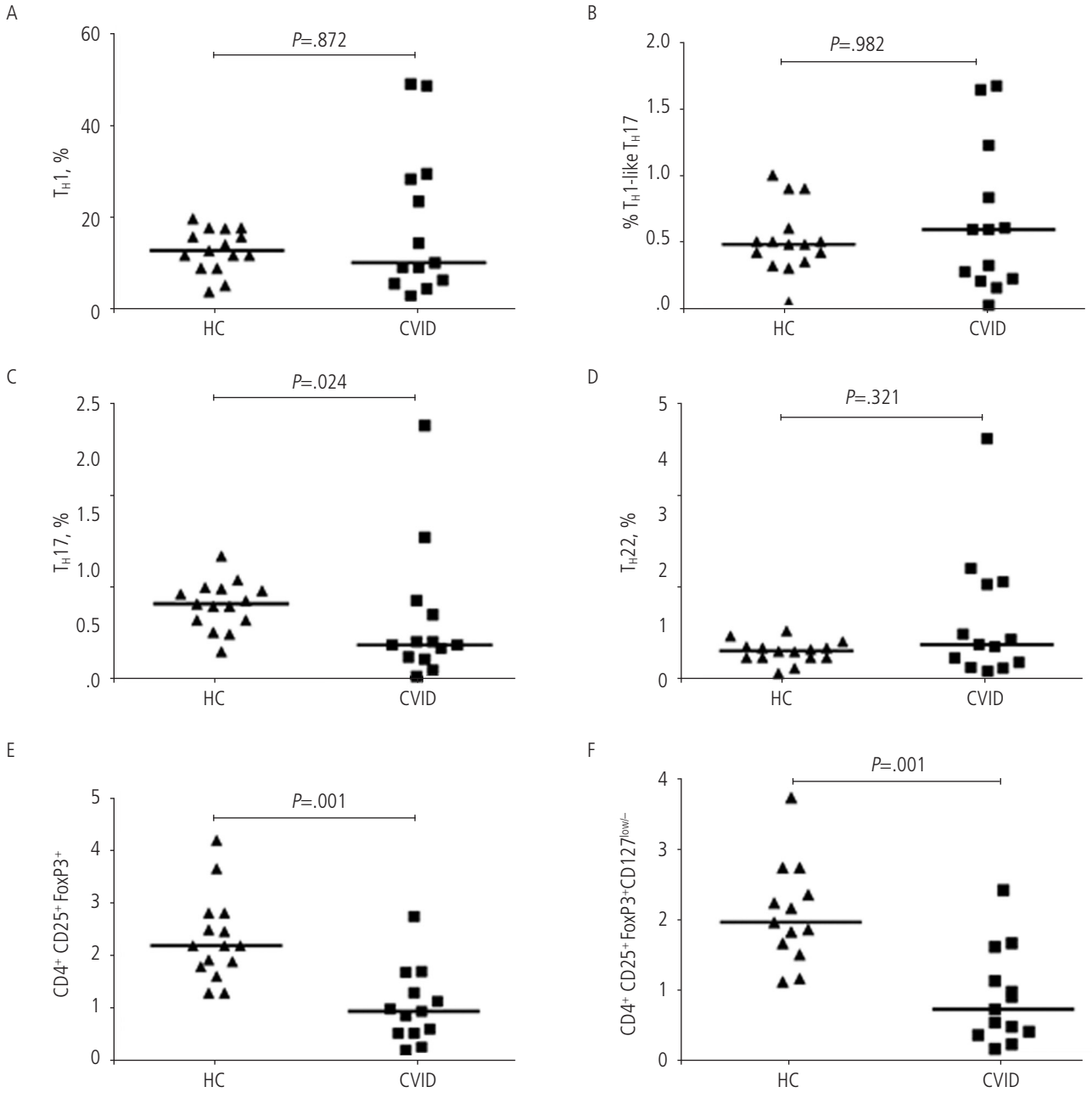

Figure 1. Quantitative analysis of different subsets of $CD4^+$ T cells. Percentage of T_H1 (A), T_H1-like T_H17 (B), T_H17 (C), T_H22 (D), Treg (E) and $CD127^{low/-}$ Treg (F) cells from patients with common variable immunodeficiency (CVID) and healthy controls (HCs) were evaluated. The median is represented by a horizontal line.

and mucosal candidiasis and biopsy-proven enteropathy were observed in 1 patient each (7.7%). A history of severe infection (meningitis, septicemia, and osteomyelitis), granuloma, autoimmunity, allergic symptoms, and malignancy were not recorded in any of these CVID patients.

Frequency of T_H Subsets in CVID Patients

In order to compare the distribution of peripheral T_H subsets in CVID patients and HCs, we examined the relative abundance and absolute counts of T_H1 (IFN-γ^+ IL-17^-), T_H17

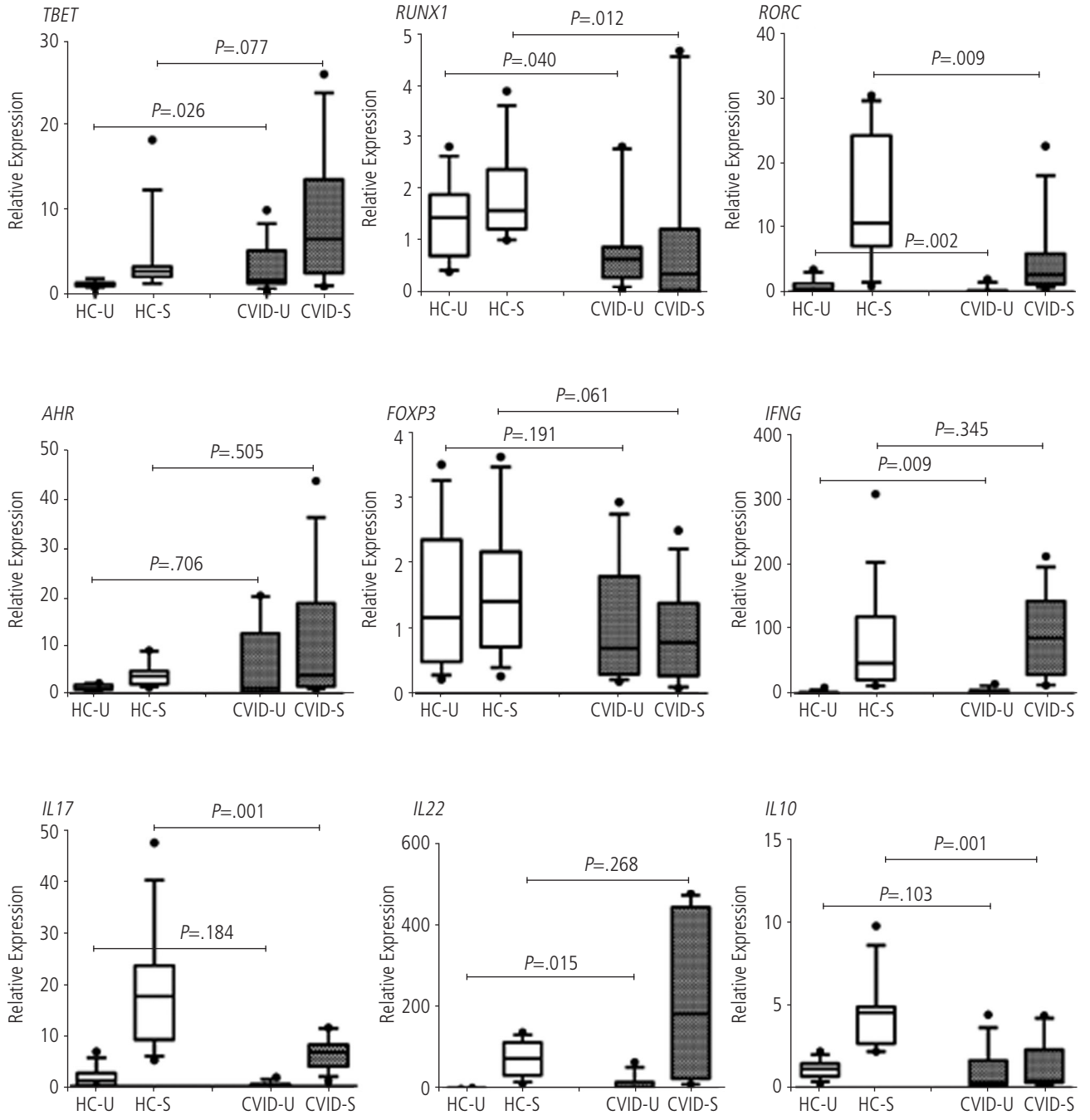

Figure 2. CD4+ T-cell gene expression in patients with common variable immunodeficiency (CVID) and healthy controls (HCs). Comparison of *TBET, RORC, AHR, RUNX1, FOXP3, IFNG, IL17, IL22*, and *IL10* gene expression in the CD4+ T cells of CVID patients and HCs using quantitative RT-PCR. The median is represented by a horizontal line, the interquartile range (IQR) by a box, and the 10th and 90th percentiles by whiskers. Outlying data (•) lie beyond the end of the whiskers. U indicates unstimulated; S, stimulated.

(IFN-γ^- IL-17^+), T_H1-like T_H17 (IFN-γ^+ IL-17^+), and T_H22 (IFN-γ^- IL-17^- IL-22^+) $CD4^+$ T cells based on their specific cytokine patterns after stimulation with PMA and ionomycin. The gating procedure and typical dot plots in defining the expression of circulating T_H subsets are depicted in Figure S1A-C. The median percentages of $CD4^+$ T cells in the CVID patients were significantly lower than in the HC group (35.30% [18.90%-39.10%] vs 46.70% [42.00%-50.20%], *P*=.001). Although 7 of 13 patients (53.8%) had reduced $CD4^+$ T-cell counts (defined as $CD4^+$ T-cell counts 2SD lower than the mean of the HC group), profound $CD4^+$T-cell deficiency (defined as $CD4^+$ T cells/μL at 2-6 years <300, 6-12 years <250, and >12 years <200) [9] was found in 1 CVID patient.

There were no significant differences in the median percentages of T_H1, T_H1-like T_H17, and T_H22 cells in CVID patients compared with HCs (9.32% [5.14%-28.20%] vs 12.00% [8.22%-16.90%], *P*=.872; 0.59% [0.21%-1.02%] vs 0.48% [0.35%-0.60%], *P*=.982; and 0.64% [0.26%-1.78%] vs 0.52% [0.40%-0.60%], *P*=.321, respectively). However, the median percentages of T_H17 cells in CVID patients was significantly lower than in HCs (0.30% [0.18%-0.64%] vs 0.71% [0.53%-0.82%], *P*=.024) (Figure 1A-D). Similarly, the absolute counts of $CD4^+$ T cells and T_H17 in CVID patients were significantly lower than that in the HCs (*P*=.003 and *P*=.001, respectively) (Table S3).

In the HC group, the percentages of T_H22 cells were positively correlated with T_H1, T_H17, and T_H1-like T_H17 cells (*P*=.022, r=0.584; *P*=.055, r=504; *P*=.042, r=0.531, respectively), and the percentages of T_H17 cells were correlated with T_H1-like T_H17 cells (*P*=.050, r=0.513). However, the pattern was not observed in CVID group except for the correlation between the percentages of T_H1 and T_H1-like T_H17 cells (*P*=.051, r=0.551). When patients were classified based on whether or not they had skin infection, the frequency of T_H1 and T_H17 cells was higher in CVID patients with skin infection than in those without (median [IQR], 35.40% [12.69%-48.30%] vs. 8.38% [4.24%-20.60%], *P*=.045, and 0.52% [0.30%-1.91%] vs. 0.27% [0.12%-0.45%], *P*=.089).

Decreased Percentage of Treg Cells in CVID Patients

The frequency of Treg cells ($CD4^+$ $CD25^+$ $FoxP3^+$) in the peripheral blood of CVID patients and HCs was evaluated. We further analyzed the percentages of CD127 expression on Treg cells. The gating procedure and typical dot plots for the expression of circulating Treg cells are depicted in Figure S1D-F. We found that both total Treg and $CD127^{low/-}$ Treg cell percentages were significantly lower in CVID patients than in HCs (median [IQR], 0.85% [0.43%-1.39%] vs 2.10% [1.70%-2.72%], *P*<.001; and .66% [0.32%-1.30%] vs 1.90% [1.52%-2.48%], *P*=.001, respectively) (Figure 1E-F). The mean fluorescence intensity (MFI) of FoxP3 protein was lower in CVID patients than in HCs (12.10 [8.55-13.10] vs 17.90 [12.55-24.20], *P*=.018). There were no significant correlations between the frequencies of total Tregs and $CD127^{low/-}$ Treg cells with T_H subsets in CVID patients. Patients with chronic diarrhea had lower $CD127^{low/-}$ Treg cells than patients without chronic diarrhea (0.38% [0.20%-0.91%] vs 0.91% [0.75%-1.97%], *P*=.040). We further analyzed the absolute counts of Treg cells and found that the total numbers of Treg cells in CVID patients were significantly lower than that in the HCs (*P*<.001) (Table S3).

Cytokines and Transcription Factor mRNA Expression

Transcription levels of *TBET, RORC, AHR, RUNX1, FOXP3* and of the cytokine genes *IFNG, IL17, IL22*, and *IL10* were evaluated for the $CD4^+$ T cells of CVID patients and HCs with and without stimulation by anti-CD3 and anti-CD28. The results showed that in the absence of stimulation, the gene expression levels of *TBET, IFNG*, and *IL22* in CVID patients were significantly higher than in HCs, whereas the transcription levels of *RORC* and *RUNX1* were significantly lower than in HCs (Figure 2). Similarly, after stimulation, the expression levels of *RORC, RUNX1, IL17*, and *IL10* in CVID patients were significantly lower than in the HC group (Figure 2). Moreover, after stimulation of $CD4^+$ T cells, the median fold change in TBET was higher, while *FOXP3* was lower in CVID patients than in HCs. However, the differences were not significant.

Cytokine Production by CD4⁺ T Cells

To test the function of different subsets of $CD4^+$ T cells, we measured the concentrations of the corresponding predominant cytokines—IFN-γ, IL-17, IL-22, and IL-10—in cell culture supernatants. There was no significant difference in cytokine production in the absence of stimulation (Figure 3A). Following stimulation with anti-CD3 and anti CD-28 mAbs, production of IFN-γ was slightly higher in CVID patients than in HCs (median [IQR], 1693.5 [962.7-3686.8] vs 945.1 [886.6-1066.5] pg/mL; *P*=.068) (Figure 3B). Moreover, concentrations of IL-17, IL-22, and IL-10 were lower in the cell culture supernatants of stimulated $CD4^+$ T cells from CVID patients than in those from HCs (median [IQR], 48.3 [39.6-204.2] vs 495.4 [337.5-973.2] pg/mL, *P*=0.004; 251.0 [84.5-592.5] vs 413.0 [305.0-605.5] pg/mL, *P*=0.248; and 196.4 [24.0-331.7] vs. 415.2 [353.1-532.4] pg/mL, *P*=0.021, respectively).

Finally, in CVID patients with the lymphoproliferative clinical phenotype, the frequency of $CD4^+$ T cells, T_H1, T_H17, T_H22, and Treg cells and their determinant cytokines (IFN-γ, IL-17, IL-22, and IL-10) was lower when T_H1-like T_H17 cell levels were higher than those of CVID patients with only the infectious clinical phenotype. However, the differences were not significant.

Discussion

Several studies reported that the typical clinical features of CVID are respiratory tract infection, enteropathy, and lymphoproliferative and autoimmune disorders [6,17,18]. In a study by Resnick et al [17], 94% of 473 patients had a history of infection, mostly respiratory tract infections, although autoimmune disease was also diagnosed in 28.6%, bronchiectasis in 11.2%, and enteropathy in 15.4%. In a study by Chapel et al [6] enteropathy was reported in 9%, autoimmunity in 21.6%, and splenomegaly in 30% of patients. Finally, Gathmann et al [19] reported that the clinical picture

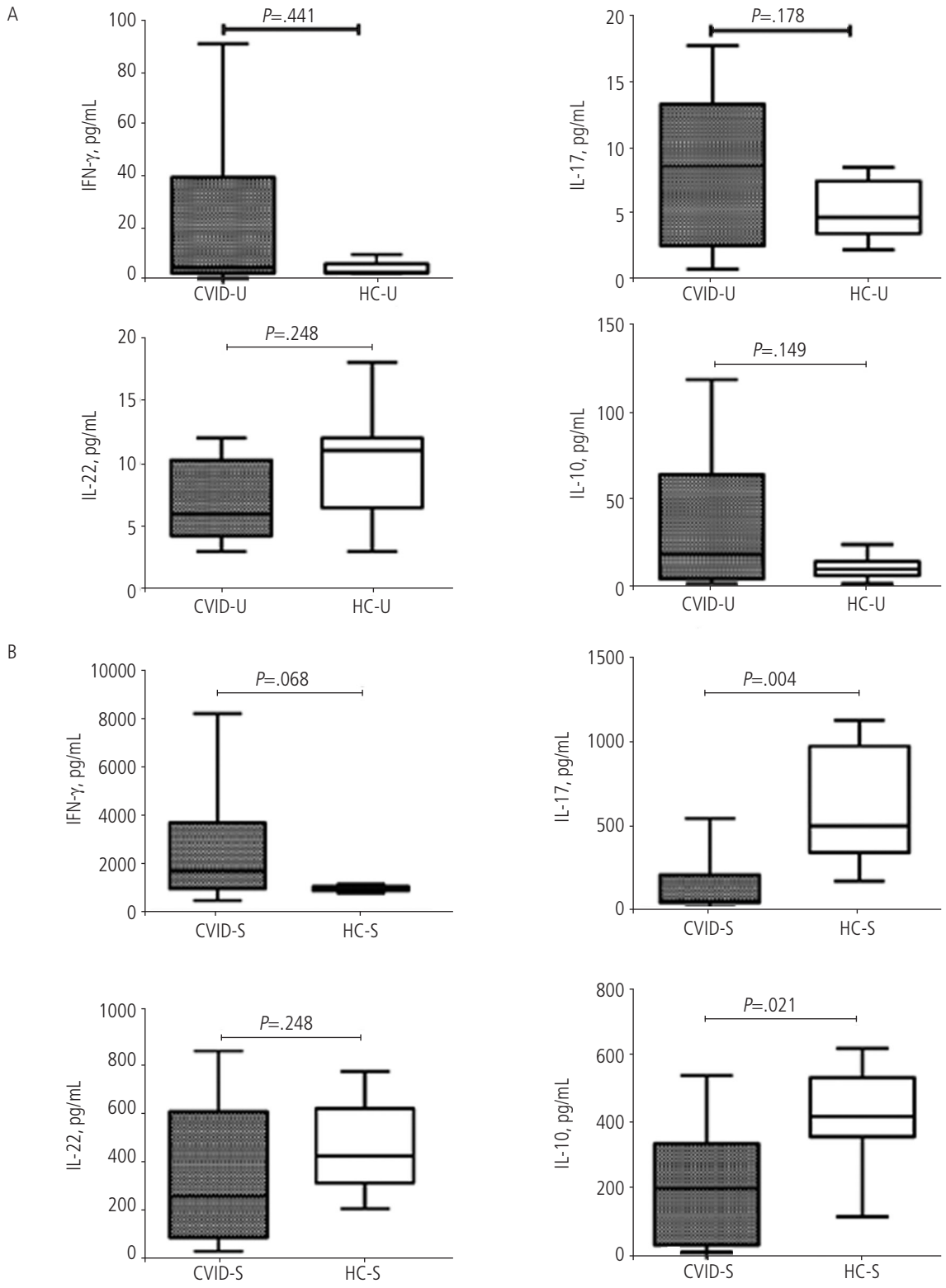

Figure 3. Cytokine secretion by CD^+ T cells in patients with common variable immunodeficiency (CVID) and healthy controls (HCs). Comparison of IFN-γ, IL-17, IL-22, and IL-10 production by $CD4^+$ T cells of CVID patients and HC, with (A) or without (B) stimulation by anti-CD3 and anti-CD28 mAbs. The median is represented by horizontal line, the interquartile range by box, and the 10th and 90th percentiles by whiskers. Outlier symbol (•) showed a data beyond the end of the whiskers. U indicates unstimulated; S, stimulated.

of 2212 CVID patients comprised autoimmunity in 29%, splenomegaly in 26%, bronchiectasis in 23%, and enteropathy in 9%. However, none of these studies performed a full genetic investigation using next-generation sequencing. Thus, the clinical phenotypes could be biased in these studies by the finding of monogenic PID mimicking the immunologic profile of CVID. In the present study, the clinical complications of CVID patients after excluding all known monogenic PIDs were less numerous than in the abovementioned studies: 77% had a history of respiratory tract infection, 30.8% had splenomegaly, and 7.7% had biopsy-proven enteropathy. None of the patients had a history of autoimmunity. However, in our previous study on non–genetically evaluated CVID patients, there was a higher frequency of clinical complications, including autoimmunity in 40.3%, enteropathy in 15.3%, and splenomegaly in 40.3% [11]. The fewer clinical symptoms (particularly autoimmunity) in our study are evident in comparison with the previous study. We suggest that the lower frequency of clinical symptoms in our study is related to differences in the inclusion criteria. Obviously, mutation analysis reveals a higher percentage of monogenic disorders such as LRBA, CD27, and CD70 deficiencies, which have more severe clinical presentations, including autoimmunity, enteropathy, and lymphoproliferative diseases [20-22]. Therefore, the remaining CVID patients in whom mutations are not found are patients with milder clinical symptoms. Importantly, the lymphoproliferative phenotype was the major clinical complications among patients whose diagnosis remained unresolved.

In the present study, $CD4^+$ T-cell counts were low in 53.8% of unresolved cases of CVID. Similar findings have been reported elsewhere, and imbalance of some subsets was correlated with severity of immune dysregulation (autoimmunity, lymphoproliferative disorder, and organ inflammation) [9,11]. We showed no significant differences in the percentages of T_H1, T_H1-like T_H17, and T_H22 cells in CVID patients compared with HCs, although we did find differences for T_H17. In a study by Coraglia et al [23], follicular $CD4^+$ T helper cells were more numerous in CVID patients than in HCs, particularly in those with severe clinical features of inflammation and autoimmunity. Moreover, it has been reported that in CVID patients with immune dysregulation, $CD4^+$ T-cell differentiation was strongly skewed toward a T_H1 phenotype, consistent with the higher IFN-γ production and impaired IL-4 signaling in $CD4^+$ T cells [24-26]. Finally, Kutukculer et al [27] reported that T_H1 cells are more involved in the pathogenesis of CVID than T_H2 cells. As mentioned, in contrast with previous studies, we did not observe any differences in T_H1 frequency in CVID compared with HCs, although in previous studies, the higher frequency of T_H1 was correlated with the severe clinical features of inflammation and autoimmunity, which were rare in our CVID patients. Although the lack of similar studies on the frequency of T_H1-like T_H17 and T_H22 cells in CVID patients prevents us from comparing our findings, several studies reported a higher frequency of this subset in nonimmunodeficient patients with autoimmunity and enteropathy [28,29]. Recently, it was reported that pathogenic T_H17 cells can give rise to T_H1-like T_H17 cells that are implicated in the development of autoimmune diseases and enteropathy [30]. On the other hand, in a previous study, we found that LRBA patients have higher T_H1-like T_H17 cell counts than HCs and that the frequency of this subset in LRBA patients with autoimmunity and enteropathy was higher than in patients without these complications (unpublished data). Therefore, homeostasis of these subsets in the CVID patients we report corresponds to the rare episodes of autoimmunity and enteropathy. We found that the percentages of T_H17 cells, transcripts of *RORC*, and *IL17* in CVID were significantly lower than in HCs. This finding has also been reported in previous studies. Barbosa et al [31] reported a reduced frequency of circulating T_H17 cells in CVID patients. Ganjalikhani-Hakemi et al [32] found that the transcript levels of IL17 and RORC2 in CVID patients were considerably lower than in HCs. Moreover, Berron-Ruiz et al [33] observed lower levels of IL-17 in CVID patients. However, the high frequency of T_H17 cells and IL-17 was associated with autoimmunity in several studies. This phenomenon is absent in CVID, probably owing to high plasticity and/or apoptosis of T_H17 cells in the special condition of CVID.

Reduced frequency of Treg cells is another defect of CVID patients [26]. Arandi et al [12] showed that Treg frequency and suppressive function were impaired in CVID patients. Yu et al [34] also reported a lower suppressive function of Tregs in CVID patients with autoimmune disease than in CVID patients with no autoimmune disease. In the present study, consistent with previous reports, we found that total Treg cell and $CD127^{low/-}$ Treg cell counts were significantly lower in CVID patients without monogenic disorders. However, Coraglia et al [23] proposed that Treg percentages were similar in CVID patients and in HCs. On the other hand, the correlation between Treg deficiency and clinical manifestation is contradictory in few studies. Kofod-Olsen et al [35] observed that the frequency of Tregs is correlated with clinical manifestations, including autoimmunity, and splenomegaly. Melo et al [26] also stratified CVID patients based on autoimmune status but did not record any association with Treg frequency. Kutukculer et al [27] reached the same conclusion in their study on the percentages and absolute numbers of Treg cells, which did not differ significantly between CVID patients and HCs, or between severe and moderate forms of CVID.

In conclusion, apart from antibody deficiency, Treg and T_H17 subset deficiencies are an intrinsic characteristic of CVID patients with no known monogenic disorder confirmed in whole exome sequencing. Given that several potential polygenic and epigenetic etiologies may underlie pathogenesis in these patients, other next-generation sequencing technologies such as RNA sequencing and epigenetic studies (DNA methylation, histone modification, and noncoding RNA-associated gene silencing) can further contribute to our understanding of the complexities of CVID.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

1. Azizi G, Abolhassani H, Asgardoon MH, Alinia T, Yazdani R, Mohammadi J, et al. Autoimmunity in common variable immunodeficiency: epidemiology, pathophysiology and management. Expert Rev Clin Immunol. 2017;13:101-15.
2. Conley ME, Notarangelo LD, and Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol. 1999;93:190-7.
3. Yazdani R, Abolhassani H, Rezaei N, Azizi G, Hammarstrom L, and Aghamohammadi A. Evaluation of Known Defective Signaling-Associated Molecules in Patients Who Primarily Diagnosed as Common Variable Immunodeficiency. Int Rev Immunol. 2016;35:7-24.
4. Yazdani R, Ganjalikhani-Hakemi M, Esmaeili M, Abolhassani H, Vaeli S, Rezaei A, et al. Impaired Akt phosphorylation in B-cells of patients with common variable immunodeficiency. Clin Immunol. 2017;175:124-32.
5. Azizi G, Abolhassani H, Mahdaviani SA, Chavoshzadeh Z, Eshghi P, Yazdani R, et al. Clinical, Immunological, Molecular Analyses and Outcomes of Iranian Patients with LRBA Deficiency: A Longitudinal Study. Pediatr Allergy Immunol. 2017;28:478-84.
6. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood. 2008;112:277-86.
7. Azizi G, Ahmadi M, Abolhassani H, Yazdani R, Mohammadi H, Mirshafiey A, et al. Autoimmunity in Primary Antibody Deficiencies. Int Arch Allergy Immunol. 2016;171:180-93.
8. Yazdani R, Heydari A, Azizi G, Abolhassani H, and Aghamohammadi A. Asthma and Allergic Diseases in a Selected Group of Patients With Common Variable Immunodeficiency. J Investig Allergol Clin Immunol. 2016;26:209-11.
9. Azizi G, Rezaei N, Kiaee F, Tavakolinia N, Yazdani R, Mirshafiey A, et al. T-Cell Abnormalities in Common Variable Immunodeficiency. J Investig Allergol Clin Immunol. 2016;26:233-43.
10. Azizi G, Hafezi N, Mohammadi H, Yazdai R, Alinia T, Tavakol M, et al. Abnormality of regulatory T cells in common variable immunodeficiency. Cell Immunol. 2016;315:11-7.
11. Azizi G, Abolhassani H, Kiaee F, Tavakolinia N, Rafiemanesh H, Yazdani R, et al. Autoimmunity and its association with regulatory T cells and B cell subsets in patients with common variable immunodeficiency. Allergol Immunopathol (Madr). 2018;46:127-35.
12. Arandi N, Mirshafiey A, Abolhassani H, Jeddi-Tehrani M, Edalat R, Sadeghi B, et al. Frequency and expression of inhibitory markers of CD4(+) CD25(+) FOXP3(+) regulatory T cells in patients with common variable immunodeficiency. Scand J Immunol. 2013;77:405-12.
13. Oraei M, Aghamohammadi A, Rezaei N, Bidad K, Gheflati Z, Amirkhani A, et al. Naive CD4+ T cells and recent thymic emigrants in common variable immunodeficiency. J Investig Allergol Clin Immunol. 2012;22:160-7.
14. Aghamohammadi A, Mohammadinejad P, Abolhassani H, Mirminachi B, Movahedi M, Gharagozlou M, et al. Primary immunodeficiency disorders in Iran: update and new insights from the third report of the national registry. J Clin Immunol. 2014;34:478-90.
15. Abolhassani H, Wang N, Aghamohammadi A, Rezaei N, Lee YN, Frugoni F, et al. A hypomorphic recombination-activating gene 1 (RAG1) mutation resulting in a phenotype resembling common variable immunodeficiency. J Allergy Clin Immunol. 2014;134:1375-80.
16. Fang M, Abolhassani H, Lim CK, Zhang J, and Hammarstrom L. Next Generation Sequencing Data Analysis in Primary Immunodeficiency Disorders - Future Directions. J Clin Immunol. 2016;36:68-75.
17. Resnick ES, Moshier EL, Godbold JH, and Cunningham-Rundles C. Morbidity and mortality in common variable immune deficiency over 4 decades. Blood. 2012;119:1650-7.
18. Yazdani R AG, Abolhassani H, Asgardoon M, Shaghaghi M, Modaresi M, Aghamohammadi A. Infectious and noninfectious pulmonary complications in patients with primary immunodeficiency disorders. J Investig Allergol Clin Immunol. 2017;27:213-24.
19. Gathmann B, Mahlaoui N, Ceredih, Gerard L, Oksenhendler E, Warnatz K, et al. Clinical picture and treatment of 2212 patients with common variable immunodeficiency. J Allergy Clin Immunol. 2014;134:116-26.
20. Azizi G, Pouyani MR, Abolhassani H, Sharifi L, Dizaji MZ, Mohammadi J, et al. Cellular and molecular mechanisms of immune dysregulation and autoimmunity. Cell Immunol. 2016; 310:14-26.
21. Alkhairy OK, Perez-Becker R, Driessen GJ, Abolhassani H, van Montfrans J, Borte S, et al. Novel mutations in TNFRSF7/CD27: Clinical, immunologic, and genetic characterization of human CD27 deficiency. J Allergy Clin Immunol. 2015;136:703-12.
22. Abolhassani H, Edwards ES, Ikinciogullari A, Jing H, Borte S, Buggert M, et al. Combined immunodeficiency and Epstein-Barr virus-induced B cell malignancy in humans with inherited CD70 deficiency. J Exp Med. 2017;214:91-106.
23. Coraglia A, Galassi N, Fernandez Romero DS, Juri MC, Felippo M, Malbran A, et al. Common Variable Immunodeficiency and Circulating TFH. J Immunol Res. 2016;2016:4951587.
24. Unger S, Seidl M, van Schouwenburg P, Rakhmanov M, Bulashevska A, Frede N, et al. The TH1 phenotype of follicular helper T cells indicates an IFN-gamma-associated immune dysregulation in patients with CD21low common variable immunodeficiency. J Allergy Clin Immunol. 2018;141:730-40.
25. Taraldsrud E, Fevang B, Jorgensen SF, Moltu K, Hilden V, Tasken K, et al. Defective IL-4 signaling in T cells defines severe common variable immunodeficiency. J Autoimmun. 2017;81:110-9.
26. Melo KM, Carvalho KI, Bruno FR, Ndhlovu LC, Ballan WM, Nixon DF, et al. A decreased frequency of regulatory T cells in patients with common variable immunodeficiency. PLoS One. 2009;4:e6269.
27. Kutukculer N, Azarsiz E, Aksu G, and Karaca NE. CD4+CD25+Foxp3+ T regulatory cells, Th1 (CCR5, IL-2, IFN-gamma) and Th2 (CCR4, IL-4, Il-13) type chemokine receptors and intracellular cytokines in children with common

variable immunodeficiency. Int J Immunopathol Pharmacol. 2016;29:241-51.
28. Azizi G, Yazdani R, and Mirshafiey A. Th22 cells in autoimmunity: a review of current knowledge. Eur Ann Allergy Clin Immunol. 2015;47:108-17.
29. Mirshafiey A, Simhag A, El Rouby NM, Azizi G. T-helper 22 cells as a new player in chronic inflammatory skin disorders. Int J Dermatol. 2015;54:880-8.
30. Harbour SN, Maynard CL, Zindl CL, Schoeb TR, and Weaver CT. Th17 cells give rise to Th1 cells that are required for the pathogenesis of colitis. Proc Natl Acad Sci U S A. 2015;112:7061-6.
31. Barbosa RR, Silva SP, Silva SL, Melo AC, Pedro E, Barbosa MP, et al. Primary B-cell deficiencies reveal a link between human IL-17-producing CD4 T-cell homeostasis and B-cell differentiation. PLoS One. 2011;6:e22848.
32. Ganjalikhani-Hakemi M, Yazdani R, Sherkat R, Homayouni V, Masjedi M, and Hosseini M. Evaluation of the T helper 17 cell specific genes and the innate lymphoid cells counts in the peripheral blood of patients with the common variable immunodeficiency. J Res Med Sci. 2014;19:S30-5.
33. Berron-Ruiz L, Lopez-Herrera G, Vargas-Hernandez A, Santos-Argumedo L, Lopez-Macias C, Isibasi A, et al. Impaired selective cytokine production by CD4(+) T cells in Common Variable Immunodeficiency associated with the absence of memory B cells. Clin Immunol. 2016;166-167:19-26.
34. Yu GP, Chiang D, Song SJ, Hoyte EG, Huang J, Vanishsarn C, et al. Regulatory T cell dysfunction in subjects with common variable immunodeficiency complicated by autoimmune disease. Clin Immunol. 2009;131:240-53.
35. Kofod-Olsen E, Jorgensen SE, Nissen SK, Westh L, Moller BK, Ostergaard L, et al. Altered fraction of regulatory B and T cells is correlated with autoimmune phenomena and splenomegaly in patients with CVID. Clin Immunol. 2016;162:49-7.

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