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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 (Th) 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. Th 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, Th17, and Treg cells were significantly reduced in CVID patients; however, Th1, Th1-like Th17, and Th22 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 Th17 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...
Helper T-Cell Subsets and Regulatory T Cells in Common Variable Immunodeficiency

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 Treg subsets and their intricate balance with Treg cells. In the present study, we evaluated the proportion and frequencies of peripheral Treg1, Treg17, Treg17, Treg22, and CD127low/− 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.

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...
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 Th1 cells, 1×10⁶ 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% CO₂ 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 Fx/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 Th1 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 Th1, Th1-like Th17, Th17, and Th22 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-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 2×10⁶/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% CO₂ 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 3×10⁶/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 used as appropriate.

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

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

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](image-url)

**Figure 1.** Quantitative analysis of different subsets of CD4+ T cells. Percentage of Th1 (A), Th1-like Th17 (B), Th17 (C), Th22 (D), Treg (E) and CD127low/– 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_{H}1 (IFN-γ IL-17), T_{H}7 (IL-22), and T_{H}9 (IL-10). The results are presented in Figure 2.

![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.](image-url)
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...between the frequencies of total Tregs and CD127 low/– Treg [12.55-24.20], in CVID patients than in HCs (12.10 [8.55-13.10] vs. 17.90 [11.55-24.20], respectively), and the percentages of TH17 cells were correlated with TH 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=0.40). 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-CD28 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=0.068) (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, Th1, Th17, Th22, and Threg cells and their determinant cytokines (IFN-γ, IL-17, IL-22, and IL-10) was lower when Th1-like Th17 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 CD4+ 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 TH1, TH1-like TH17, and TH22 cells in CVID patients compared with HCs, although we did find differences for TH17. 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 TH1 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 TH1 cells are more involved in the pathogenesis of CVID than TH2 cells. As mentioned, in contrast with previous studies, we did not observe any differences in TH1 frequency in CVID compared with HCs, although in previous studies, the higher frequency of TH1 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 TH1-like TH17 and TH22 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 TH17 cells can give rise to TH1-like TH17 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 TH1-like TH17 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 TH17 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 TH17 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, Berro-Ruiz et al [33] observed lower levels of IL-17 in CVID patients. However, the high frequency of TH17 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 TH17 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 CD127low 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. Kutukcüler 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 TH17 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.
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