The circulating T helper subsets and regulatory T cells in patients with common variable immunodeficiency with no known monogenic disease

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Running Head: T helper subsets and regulatory T cells in common variable immunodeficiency

Abstract

***Background*:** Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (PID), characterized by heterogeneous clinical manifestations and defects in B- and T- cells. In the present study, we investigated the T helper (Th) cell subsets and regulatory T (Treg) cells, and their related cytokines and transcription factors in the CVID patients with no definite genetic diagnosis.

***Methods*:** The study population comprised 13 CVID patients and 13 healthy controls (HC). Mutation analysis was done by whole exome sequencing in CVID patients to rule out monogenic PIDs. Th subsets and Treg were examined by flow cytometry. The expression of determinant cytokines (IFN-γ, IL-17, IL-22, and IL-10), and cell subset specific transcription factors were evaluated before and after stimulation.

***Results*:** The main clinical presentations of these patients were infections only and lymphoproliferations phenotypes, but no autoimmune and allergy phenotype were recorded. The frequencies of $CD4^+$ T cells, Th17, and Treg cells were significantly reduced in CVID patients, however the subsets of Th1, Th1-like Th17 and Th22 cells were normal. After stimulation, retinoic-acid-orphan-receptor-C (RORC), and runt-related transcription factor 1 (RUNX1), IL17, and IL10 genes' expression in CVID patients were significantly lower, in comparison to the HC. Moreover, there was a lower concentration of IL-17 and IL-10 in cell culture supernatants of stimulated $CD4^+$ T cells of CVID patients than HC.

***Conclusions*:** Our findings demonstrate that the imbalance of Th17 and Tregs could be an associated with infections only and lymphoproliferations phenotype in CVID patients without monogenic disorders.

Keywords: Common variable immunodeficiency; CVID; infection; autoimmunity; lymphoproliferations; regulatory T cell; T helper 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.

1- Introduction

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (PID), characterized by heterogeneous clinical/immunologic manifestations [1] including susceptibility to recurrent infections, hypogammaglubolinemia and reduced specific antibody response to protein and polysaccharide antigens [2]. The underlying causes of CVID are largely obscure. Genetic mutations can be identified as the cause of disease in approximately 10-20% of patients depends on the ethnicity and population structure of the cohort [3]. These include mainly the members of the B cell co-receptor complex, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B cell-activating factor receptor (BAFFR), inducible co-stimulator (ICOS) and lipopolysaccharide responsive beige-like anchor (LRBA) genes. Although these new monogenic defects share clinical phenotypes with CVID, they could be considered as distinct PID entities with a CVID-like phenotype [3-5]. However, more than 80% of clinically diagnosed CVID patients are lacking a definite molecular genetic diagnosis or other causal pathogenesis for their disease.

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 involve in the pathogenesis of immune dysregulation in CVID patients. The T-cell defects that characterized in CVID included $CD4^+$ T-cell deficiency, defect in regulatory T cell (Tregs) counts and function, decreased lymphocyte proliferation, uncontrolled T-cell polarization, elevated levels of T-cell activation markers, and abnormality in cytokine production [9-13].

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Although the abnormality of $CD4^+$ T cell has been linked to CVID clinical presentations including lymphoproliferative and autoimmune disorders [11], there is no study at present on the frequencies and functional status of Th subsets and their intricate balance with Treg cells. In the present study, we evaluated the proportion and frequencies of peripheral Th1, Th1-like, Th17, Th22 and $CD127^{low/-}$ Treg cells as well as their determinant cytokines and transcriptional factors in CVID patients with no definitive genetic diagnosis.

2- Subjects and methods

2.1. Subjects

The study population comprised available CVID patients that referred to PID Clinic at Children's Medical Center affiliated to Tehran University of Medical Sciences, Tehran-Iran. Thirteen healthy individuals with no history of immune related disorders (e.g. severe infection, allergy, autoimmunity or malignancy) were selected as 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. The demographic and clinical data of patients were collected from the Iranian national registry of PID patients [14] updating by monthly visits of patients, and reviewed completely 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 obtaining of intravenous Ig (IVIg).

2.2. Whole exome sequencing (WES)

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 WES designed by BGI-Shenzhen/Karolinska Institute as described previously [16]. Patients with a tentative diagnosis of CVID and an identified mutation within known 373 monogenic PID genes (**Table S1**) were

excluded from this study [15, 16]. Therefore 13 CVID patients remaining without definitive genetic diagnosis were recruited to this study.

2.3. Cell isolation and purification of $CD4^+$ T cell

The blood samples were collected four weeks after IVIg infusion in heparin-containing tubes. Lymphocyte counts were performed using the Sysmex KX-21N (Sysmex Corporation, Kobe, Japan). Peripheral blood mononuclear cells (PBMCs) were obtained from both patients and controls using Lymphocyte Separation Media (Lymphosep, Biosera, France) and re-suspended in the RPMI medium (Lymphosep, Biosera) supplemented with 10% fetal bovine serum (Lymphosep, Biosera), penicillin (100 IU), and streptomycin (100 μg/mL) (Biosera, Ringmer, East Sussex, UK) for immunophenotyping and $CD4^+$ T cell isolation. The viability of isolated PBMCs was more than 97 % as assessed by Trypan blue viability test. $CD4^+$ T cells were purified from PBMCs with a human $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany) by depletion of non-$CD4^+$ T cells (negative selection). The purity of $CD4^+$ T cells was routinely more than 95% based on flow cytometric analysis.

2.4. Flow cytometric analysis

For intracellular staining of Th cells, 1×10^6 PBMCs were stimulated with Phorbol myristate acetate (PMA, 50 ng/mL, Sigma, USA) and ionomycin (1 μg/mL, Sigma, USA) for 5 h in RPMI medium at 37 °C in a 5% CO_2 humidified atmosphere in the presence of Brefeldin A (BFA, 10 μg/mL, eBioscience, USA). The stimulated cells were washed with cold PBS and cell surface staining Abs (anti-CD4 PerCP-cy5.5, clone OKT4, eBioscience) were added and incubated in the dark at 4 °C for 30 min. Alternatively, for evaluation of Tregs, unstimulated PBMCs were stained using surface Abs (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 min. Following surface staining, the cells were washed twice, fixed and permeabilized with Fix/Perm buffer and suspended in permeabilization buffer (eBioscience). Then intracellular cytokine staining Abs (anti-IFN-γ FITC [clone CZ-4], anti-IL-17 PE [clone eBio64DEC17] and anti-IL-22 APC [clone IL22JOP] for evaluation of Th subsets and anti-FoxP3 PE [Forkhead Box P3, clone 236A/E7] for evaluation of Treg, were added and incubated at room temperature for 30 min. Cells were washed with permeabilization buffer, re-suspended in cold staining buffer, and determined by BD FACSCalibur Flow Cytometer (BD Biosciences, USA). Lymphocytes were gated on forward and side scatters' profiles and analyzed using FlowJo software (Tree star, USA). The percentage of Th1, Th1-like Th17, Th17 and Th22 lymphocytes was acquired by calculating 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, respectively. The percentage of Treg lymphocytes was acquired by calculating the percentage of $CD25^+FoxP3^+$ cells within a $CD4^+$ population. The 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.

2.5. 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 pre-coated with 3 μg/mL anti-CD3 mAb. Moreover, 2 μg/mL anti-CD28 mAb was added concurrently and incubated in RPMI medium and 37 °C in a 5% CO_2 humidified atmosphere. Stimulated cells were collected after 18 h and washed twice with PBS. For RT-PCR, total RNA was extracted from cultured cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) and reversed transcribed into cDNA by Takara kit (Takara, Japan) according to the manufacturer's instructions with

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some modifications. The expression levels of *IFNG*, *IL17*, *IL22*, *IL10*, T-box transcription factor (*TBET*) and Runt-related transcription factor 1 (*RUNX1*), RAR-related orphan receptor C (*RORC*), Aryl hydrocarbon receptor (*AHR*) and *FOXP3* genes were measured by 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).

2.6. Cytokine assay

$CD4^+$ T cells were harvested and brought to a final concentration of 3×10^5/mL in 24-well plates and subsequently were stimulated by anti-CD3 mAb and anti-CD28 mAb using the same method mentioned in the previous section. Unstimulated cells were used as controls for each experiment. Supernatants were collected after 48 hours, and cytokines' 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 for IL-10 was 2 pg/mL, for IFN-γ and IL-17 was 4 pg/mL and for IL-22 was 8 pg/mL.

2.7. Statistical analysis

Values were expressed as frequency (number and percentage), and median (IQR, presented as a range with 75^{th}-25^{th} percentiles) as appropriate. Shapiro-Wilks test used to check the normality assumption for the variable; so according to the establishment of assumptions, parametric or nonparametric test was done. Statistical analyses were performed using the SPSS software package, version 22 (SPSS Inc., Chicago, IL, USA).

3- Results

3.1. Characteristics and clinical phenotypes of CVID patients

To determine the frequency of different subsets of $CD4^+$ T cells, a total of 13 (6 males and 7 females) Iranian CVID patients without genetic diagnosis after WES were compared with 13 sex-age matched HCs. The demographic and clinical characteristics of patients are summarized in **Table 1**. The first presentation for immunodeficiency in 11 (84.6%) patients was an infection, and in 2 (15.4%) patients were chronic diarrhea. Totally, 10 (77%) of CVID patients had a history of respiratory tract infections (RTIs), and 4 (30.8%) patients had skin infections. Lymphoproliferative disorders was observed in 7 (53.8%) patients, bronchectasis, arteritis and failure to thrive each in 2 (15.4) patients, mucosal candidiasis and biopsy-proven enteropathy each in 1 (7.7%) patients, while history of severe infections (meningitis, septicemia and osteomyelitis), granuloma, autoimmunity, allergic symptoms and malignancy were not recorded in any of these CVID patients.

3.2. The frequency of Th subsets in CVID patients

In order to compare the distribution of peripheral Th subsets in CVID patients and HC, we examined the relative abundance and absolute counts of Th1 (IFN-γ^+ IL-17^-), Th17 (IFN-γ^- IL-17^+), Th1-like Th17 (IFN-γ^+ IL-17^+), and Th22 (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 Th subsets are depicted in **Figure S1A-C**. The median percentages of $CD4^+$ T cells in the CVID patients were significantly lower than that in the HC group [35.30 (18.90-39.10) vs. 46.70 (42.00-50.20) %, P=0.001]. Although 7 of 13 patients (53.8%) has reduced $CD4^+$ T cell counts (defined as $CD4^+$ T-cell counts: 2SD lower than mean of control group), but 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 defined in one CVID patient.

There was no significant differences in the median percentages of Th1, Th1-like Th17 and Th22 cells in CVID patients compared to HCs [9.32 (5.14-28.20) vs. 12.00 (8.22-16.90) %, *P*=0.872; 0.59 (0.21-1.02) vs. 0.48 (0.35-0.60) %, *P*=0.982; and 0.64 (0.26-1.78) vs. 0.52 (0.40-0.60) %, *P*=0.321, respectively]. However, the median percentages of Th17 cells in CVID was significantly lower than that in HCs [0.30 (0.18-0.64) vs. 0.71 (0.53-0.82) %, *P*=0.024] (**Figure 1A-D**). Similarly, the absolute counts of $CD4^+$ T cells and Th17 in the CVID patients were significantly lower than that in the HC (*P*=0.003 and *P*=0.001, respectively) (**Table S3**).

In HC group, the percentages of Th22 cells were positively correlated with Th1, Th17 and Th1-like Th17 cells (*P*=0.022, r=0.584; *P*=0.055, r=504; *P*=0.042, r=0.531, respectively), and the percentages of Th17 cells were correlate with Th1-like Th17 cells (*P*=0.050, r=0.513). However, the pattern not observed in CVID group except for correlation between the percentages of Th1 and Th1-like Th17 cells (*P*=0.051, r=0.551). When patients were divided based on whether or not has skin infection, the frequency of Th1 and Th17 cells was higher in CVID patients with skin infection than those without [median (IQR): 35.40 (12.69-48.30) vs. 8.38 (4.24-20.60), *P*=0.045 and 0.52 (0.30-1.91) vs. 0.27 (0.12-0.45), *P*=0.089].

3.3. Decreased percentage of Treg cells in CVID patients

The frequency of Treg cells ($CD4^+$ $CD25^+$ $FoxP3^+$) in peripheral blood of CVID patients and HC was evaluated. We further analyzed the percentages of CD127 expression on Treg cells. The gating procedure and typical dot plots in defining the expression of circulating Treg cells are depicted in **Figure S1D-F**. We found that both total Treg cells and $CD127^{low/-}$ Treg cells were significantly lower in CVID patients

than HC [median (IQR): 0.85 (0.43-1.39) vs. 2.10 (1.70-2.72) %, *P*<0.001; and 0.66 (0.32-1.30) vs. 1.90 (1.52-2.48) %, *P*=0.001, respectively] (**Figure 1E-F**). The mean fluorescence intensity (MFI) of FoxP3 protein were decreased in CVID patients compared to those in HC [12.10 (8.55-13.10) vs. 17.90 [12.55-24.20], *P*=0.018). There was no any significant correlation between the frequencies of total Tregs and $CD127^{low/-}$ Treg cells with Th subsets in CVID patients. Patients with chronic diarrhea has lower $CD127^{low/-}$ Treg cells than the patients without chronic diarrhea [0.38 (0.20-0.91) vs. 0.91 (0.75-1.97), *P*=0.040]. We further analyzed the absolute counts of Treg cells, and found the total numbers of Treg cells in the CVID patients were significantly lower than that in the HC (*P*<0.001) (**Table S3**).

3.4. Cytokines and transcription factors mRNA expression

The transcription level of *TBET*, *RORC*, *AHR*, *RUNX1*, *FOXP3*, and cytokines *IFNG*, *IL17*, *IL22* and *IL10* genes for $CD4^+$ T-cells of CVID patients and HCs were evaluated with and without anti-CD3 and anti-CD28 stimulation. The results showed that in the absence of the stimulation, the gene expressions of *TBET*, *IFNG* and *IL22* in CVID patients were significantly higher than HCs, while the transcription level of *RORC* and *RUNX1* were significantly lower than HCs (**Figure 2**). In the same manner after stimulation, *RORC*, *RUNX1*, *IL17*, and *IL10* genes' expression in CVID patients were significantly lower, in comparison to the HC group (**Figure 2**). Moreover, after $CD4^+$ T-cells stimulation, the median fold changes of *TBET* was higher, while *FOXP3* was lower in CVID patients than HC, however, the differences were not significant.

3.5. The 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 any significant difference in cytokine

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production in the absence of stimulation (**Figure 3A**). Following stimulation with anti-CD3 and anti CD-28 mAbs, a slightly higher production of IFN-γ [median (IQR): 1693.5 (962.7-3686.8) vs. 945.1 (886.6-1066.5) pg/mL; *P*=0.068] were seen in CVID patients compared to HC (**Figure 3B**). Moreover, there was a lower concentration of IL-17, IL-22 and IL-10 in cell culture supernatants of stimulated $CD4^+$ T cells of CVID patients than HC [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 lymphoproliferation clinical phenotype, the frequency of $CD4^+$ T cells, Th1, Th17, Th22 and Treg cells and their determinant cytokines (IFN-γ, IL-17, IL-22, and IL-10) was lower when Th1-like Th17 cells was higher than that CVID patients with infectious only clinical phenotype, however, the differences was not significant.

4- Discussion

Several studies reported that the typical clinical features of CVID are RTI, enteropathy, 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 RTIs, also autoimmune disease was diagnosed in 28.6%, bronchiectasis in 11.2%, and enteropathy in 15.4% of subjects. In another study by Chapel et al. [6] enteropathy was reported in 9%, autoimmunity in 21.6%, and splenomegaly in 30% of patients, and finally Gathmann et al. [19] reported the clinical picture of 2212 CVID patients as follow; autoimmunity in 29%, splenomegaly in 26%, bronchiectasis in 23%, and enteropathy in 9% of subjects. However none of these studies performed completed genetic investigation for their patients using next generation sequencing. Thus the clinical phenotypes could be biased in these studies by PID monogenic disease mimicking CVID immunologic profile. In the present study, the clinical complications of CVID patients after excluding all PID known monogenic diseases were fewer than above studies, 77% had a history of RTIs, 30.8% of patients had splenomegaly, and 7.7% of patients had biopsy-proven enteropathy, while the history of autoimmunity was not recorded in any patients. 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 in comparison with the previous study is related to different inclusion criteria in our study. Obviously, during mutation analysis, the monogenic disorders like LRBA, CD27 and CD70 deficiencies that have more severe clinical presentations including

autoimmunity, enteropathy and lymphoproliferative diseases are detected [20-22], therefore, the rest of the CVID patients in whom mutations are not found, are those patients with milder clinical symptoms. Importantly the lymphoproliferation phenotype was the major clinical complications among these unsolved patients.

In the present study, 53.8% of unsolved CVID patients had a reduced $CD4^+$ T cell counts. This was also reported in previous studies, and imbalance of some subsets was correlated with severity of immune dysregulation (autoimmunity, lymphoproliferation, 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 to HCs, but Th17. In a study by Coraglia et al. [23] $CD4^+$ T follicular helper cells (T_{FH}) were higher in CVID patients than HCs particularity in those with severe clinical features of inflammation and autoimmunity. Moreover, it has been reported that CVID patients with immune dysregulation had a strongly skewed $CD4^+$ T-cell differentiation toward a Th1 phenotype consistent with the higher IFN-γ production and an 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 to previous studies we did not observed any differences in Th1 frequency in CVID compared to HCs, however, 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 there is no any similar study in the frequency of Th1-like Th17 and Th22 cells in CVID patients to compare our findings with their results, several studies reported a higher frequency of this subset in non immunodeficient 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 hands, in a previous study we found that LRBA patients have higher Th1-like Th17 cells than HC, also the frequency of this subset in LRBA patients with autoimmunity and enteropathy was higher than patients without these complications (unpublished data). Therefore, the homeostasis of these subsets in our CVID patients corresponds with the rare episodes of autoimmunity and enteropathy in them. We found that the percentages of Th17 cells, transcripts of *RORC*, and *IL17* in CVID were significantly lower than that in HCs. This has been also suggested in previous studies as Barbosa et al. [31] reported a reduced frequency of circulating Th17 cells in CVID patients. Ganjalikhani Hakemi et al. [32] studied the transcript levels of *IL17* and *RORC2* in CVID patients, which were strongly lower than HCs. Moreover, Berron-Ruiz et al. [33] also observed lower levels of IL-17 in CVID patients. Although, high frequency Th17 cells and IL-17 were associated with autoimmunity in several studies, however, this phenomenon is absent in CVID condition, this may be due 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 Tregs frequency and their suppressive functions were impaired in CVID patients. Yu et al. [34] also reported a lower suppressive function of Tregs from CVID subjects with the autoimmune disease compared to CVID subjects with no autoimmune disease. In the present study, similar to previous reports we found that total Treg cells and $CD127^{low/-}$ Treg cells were significantly lower in CVID patients without monogenic disorders. However, Coraglia et al. [23] proposed the percentages of Tregs were similar in CVID patients and in HCs. On the other hands, the correlation of Treg deficiency with clinical manifestation is contradictory in few studies. Kofod-Olsen et al. [35] observed that the frequencies of Tregs is correlated to

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clinical manifestations, including autoimmunity, and splenomegaly. Melo et al. [26] also stratified the CVID patients based on autoimmune status, but did not record any association with Tregs' frequency. In addition, Kutukculer et al. [27] had the same conclusion in their study on the percentages and absolute numbers of Treg cells, which did not show any significant difference neither between CVID patients and HCs, nor 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 found in WES. Due to the fact that several potential polygenic and epigenetic etiologies may be behind the pathogenesis of this group of patients, other next generation sequencing technologies such as RNA sequencing and epigenetic studies (DNA methylation, histone modification and non-coding RNA-associated gene silencing) can contribute further to our understanding of the complexities CVID.

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Table 1. Demographic data and clinical characteristics for CVID patients and HCs

Parameters	CVID (N=13)	HC (N=13)
Sex ratio (M/F)	6/7	6/7
Consanguinity; N (%)	8 (61.6)	4 (30.8)
Age at the study time; median in year (IQR)	14.0 (10.0-29.0)	14.0 (10.0-29.0)
Age at onset of symptoms; median in year (IQR)	4.0 (0.75-7.0)	-
Age at the time of diagnosis; median in year (IQR)	9.0 (7.0-24.0)	-
Delay diagnosis; median in year (IQR)	9.0 (7.0-24.0)	-
Infection only phenotype; N (%)	5 (38.5)	-
Autoimmunity; N (%)	0 (0.0)	-
Enteropathy; N (%)	1 (7.7)	-
Lymphoproliferation; N (%)	7 (53.8)	-
Allergy; N (%)	0 (0.0)	-

CVID; Common variable immune deficiency, HCs; Healthy controls, M; Male, F; Female. IQR: range with 75th and 25th percentiles. N, Count.

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 CD127$^{low/-}$ Treg (**F**) cells from CVID patients and healthy controls (HC) were evaluated. The median is represented by a horizontal line.

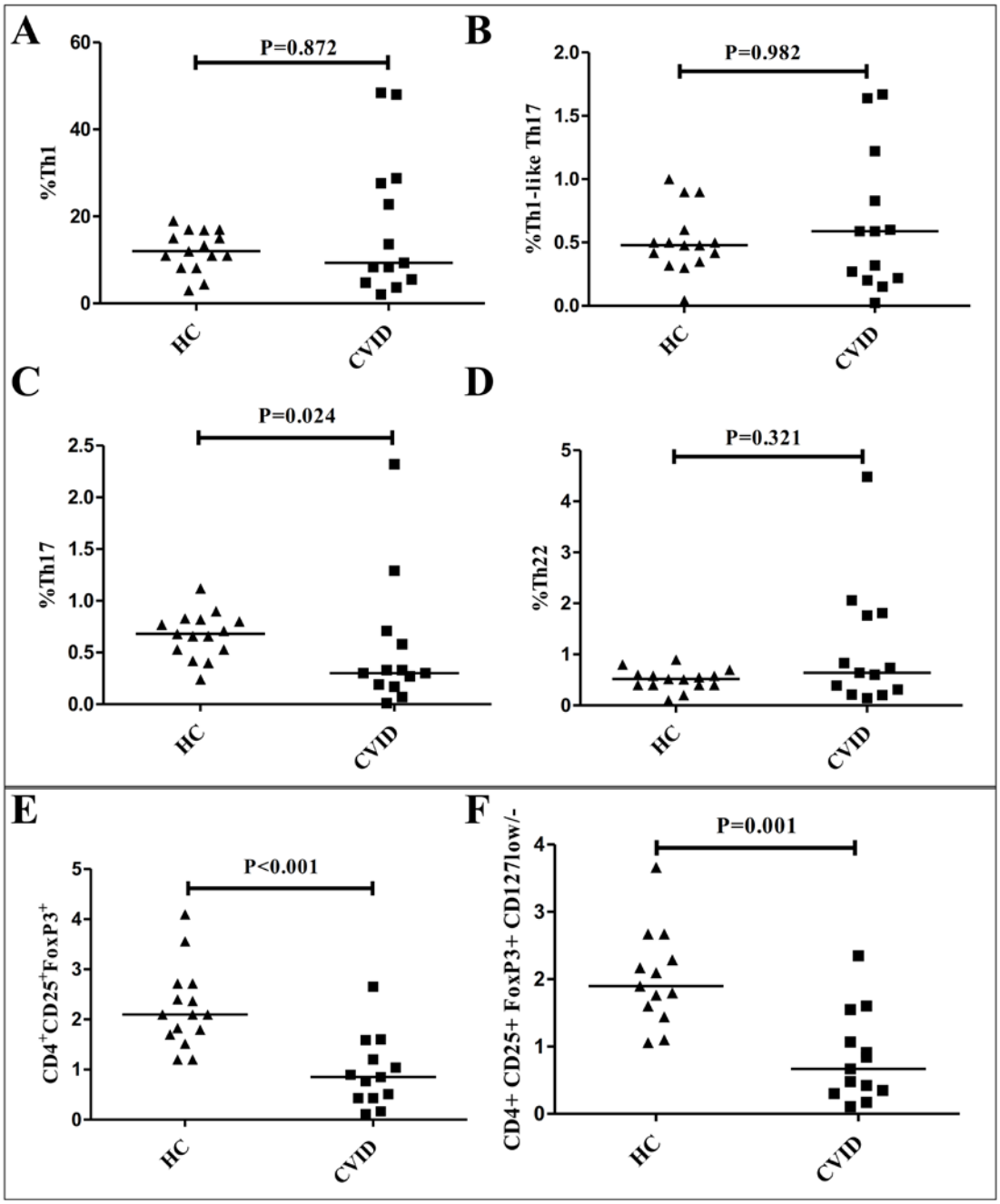

Figure 2. CD4$^+$ T cells genes expression in CVID patients and healthy controls (HC). Comparison of *TBET, RORC, AHR, RUNX1, FOXP3, IFNG, IL-17, IL-22* and *IL-10* genes expression in the CD4$^+$ T cells of the CVID patients and HC by the quantitative RT-PCR. The median is represented by a horizontal line, the interquartile range (IQR) by box, and the 10th and 90th percentiles by whiskers. Outlier symbol (•) showed a data beyond the end of the whiskers. U, un-stimulated; S, stimulated.

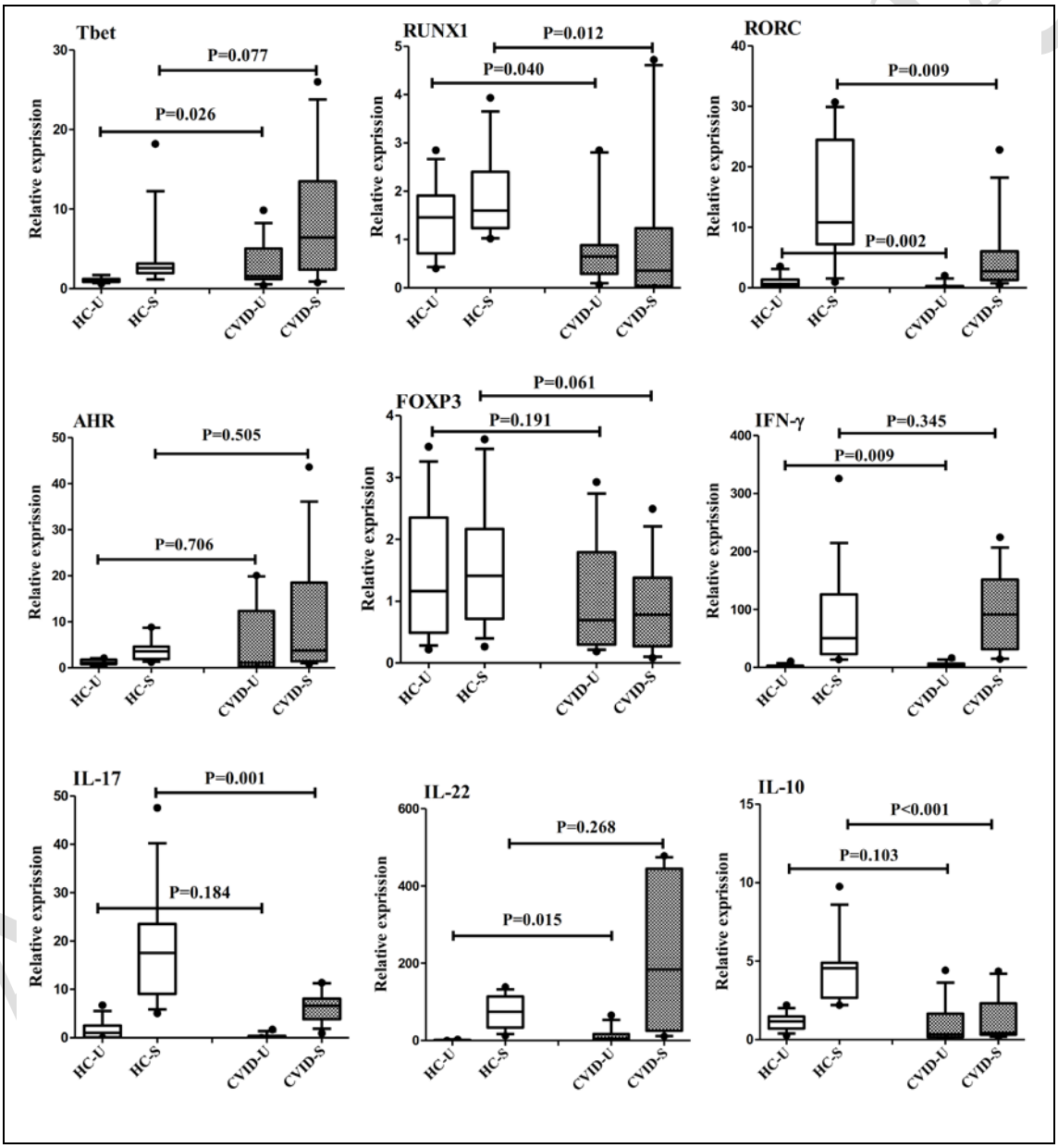

Figure 3. Cytokine secretion by CD$^+$ T cells in CVID patients and healthy controls (HC). Comparison of IFN-γ, IL-17, IL-22 and IL-10 production by CD4+ T cells of CVID patients and HC, with (A) or without (B) stimulation by anti-CD3 and anti-CD28 mAbs. The median is represented by horizontal line, 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, un-stimulated; S, stimulated.