

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

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Background: Common variable immunodeficiency (CVID) is characterized by a deficiency in the immune system with a heterogeneous collection of disorders resulting in antibody deficiency and recurrent infections. T helper 17 (Th17) cells promote B-cell survival and synergize with the B-cell activating factor to induce their differentiation into the plasma cells. A sub-population of innate lymphoid cells (ILCs) also produces interleukin 17 (IL-17). This study aimed to measure the Th17 specific genes and ILCs counts in the CVID patients in comparison with control subjects. **Materials and Methods:** Total messenger ribonucleic acid (mRNA) was extracted from the whole blood samples of 10 CVID patients and 10 healthy individuals. IL-17, retinoic acid receptor-related orphan receptor C2 (RORC2), IL-23R, and IL-9 gene expression were measured using the quantitative reverse transcriptase-polymerase chain reaction. Count of lineage negative/CD127⁺/CD90⁺ ILCs in the blood samples was performed by the flow cytometry method. **Results:** The transcript levels of IL-17 and RORC2 in CVID patients was strongly lower than control subjects ($P = 0.049$ and $P = 0.046$, respectively), but slight reduction in the IL-23R expression ($P = 0.252$) have seen in the CVID patients. Accordingly, the number of ILCs decreased significantly ($P = 0.04$). Interestingly, IL-9 mRNA level was more significantly in the CVID patients ($P = 0.001$). **Conclusion:** The results presented in this study show that the Th17 cell specific genes expression (as the determiner Th17 cells) and ILCs (another lymphoid source of IL-17) are decreased in patients with CVID and this could be an explanation for the defect of their humoral immune response. In addition, elevation of the IL-9 gene expression may shed a new light into the way toward the understanding of the mechanism of autoimmunity in the CVID patients.

Key words: Common variable immunodeficiency, innate lymphoid cells, interleukin 9, interleukin 17, interleukin 23R, T helper 17 cells

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INTRODUCTION

Common variable immunodeficiency (CVID) is the most frequent symptomatic primary immunodeficiency, which is characterized by failure of B-cell differentiation and decreased production of immunoglobulins. CVID is a combination of humoral and cell-mediated deficiency; this could be the reason for the multi-system involvement in the CVID patients, and the ineffectiveness of the intravenous immunoglobulin (IVIG) as standard therapy to prevent complications of the CVID patients.^[1] The most common symptoms are severe, recurrent and sometimes chronic bacterial infections, mostly of the respiratory and gastrointestinal tracts and an increased incidence of lymphoproliferative processes, which may predispose to neoplasia.^[2] The onset of CVID is at >2 years of age.^[3] There is no precise information on the

prevalence of CVID, but it has been anticipated to be at between 1:10,000 and 1:100,000 of the population.^[3,4] Apart from low immunoglobulin production by B-cells in CVID patients, other immunological abnormalities such as, T-cell dysfunction and monocyte/macrophage hyperactivity are observed in many patients.^[5] Among the CD4 T-cells, interleukin 17 (IL-17) producing T helper 17 (Th17) cell has a pivotal role in harmony with the natural and acquired immune responses to the extracellular bacteria and fungi^[6] and also in the pathogenesis of several autoimmune and inflammatory disorders.^[7] The retinoic acid receptor-related orphan receptor C2 (RORC2) transcription factor and IL-23R are expressed by the Th17 cells.^[7-9] Development of Th17 cells and expression of their specific cytokines IL-17A and IL-17F are promoted by the RORC2.^[10] Cytokine IL-23 (an IL-12 family member) along with IL-6 and transforming growth factor- β are the important factors for the development of the Th17 cells.^[11]

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The differentiation and survival of Th17 cells share critical clues with the B-cell differentiation and the follicular T helper (T_{FH}) subset, which was recently shown to be enriched in Th17 cells to help B-cell differentiation.^[12] B-cell differentiation in the germinal centers (GCs) is also required or may contribute to the induction and/or survival of Th17 cells.^[13]

It is reported that about 25-48% of the CVID patients are influenced by diverse autoimmune diseases (more thrombocytopenic purpura and hemolytic anemia), which are usually the first manifestation of this immune deficiency.^[3,14] A subset of lymphocytes which is called the innate lymphoid cells (ILCs), represents an emerging family of cell types which seems to have crucial roles in the tissue remodeling and in innate immunity to the pathogenic and nonpathogenic microorganisms.^[15] These cells are determined by a lymphoid morphology and a lack of receptors dependent on the RAG recombinase, encoded by the recombination activating genes.^[16] Among these ILCs, one of them overlap with the Th17 cells and produce IL-17 and IL-22.^[17] These ILCs do not express the lymphoid lineage markers (Lin^{-}), but are ROR γ t (RORC2), Thy-1 (CD90) and the IL-7 receptor- α (CD127) positives.^[17] There is some evidence to suggest that also IL-9 is produced by the Th17 cell.^[18] New reports have demonstrated that IL-9 increased in the autoimmune diseases such as, systemic lupus erythematosus (SLE), systemic sclerosis, and rheumatoid arthritis (RA).^[19-21] In general, these data implicate IL-9 as a cytokine which contribute to the autoimmune disease. Consequently, the current study aimed to evaluate the Th17 cell specific genes expression (as the determiners of Th17 cells) and IL-17-producing ILCs along with the measurement of messenger ribonucleic acid (mRNA) level of IL-9 in the CVID patients.

MATERIALS AND METHODS

Subjects

Ten CVID cases and 10 healthy controls (five males and five females per group) were enrolled in the study. The characteristics of CVID patients are summarized in Table 1.

The CVID patients were diagnosed according to the European Society for the Immunodeficiency Criteria namely: decreased serum IgG as well as IgM and/or IgA levels at least two standard deviations below the normal mean for age, impaired antibody response to vaccines, absent/low isohemagglutinins, and exclusion of defined causes of hypogammaglobulinemia.^[22] Nobody was receiving the IVIG before sampling. Healthy controls were selected from age matched volunteers without any sign, symptom and history of immunodeficiency and/or autoimmunity.

All subjects had signed informed consent for the blood sampling and processing. The ethical aspects of this study were approved by the Infectious Diseases and Tropical Medicine Research Center and Ethical Committee of Isfahan University of Medical Sciences, Isfahan, Iran (Project number : 188128).

IL-17, RORC2, IL-23R, and IL-9 were detected in the peripheral blood of patients with CVID by the real-time polymerase chain reaction (PCR). We also evaluated the frequency of the ILCs producing IL-17 in the peripheral blood of the CVID patients, using the flow cytometry method with a standard panel of monoclonal antibodies following the manufacturer's instructions.

Real-time quantitative reverse transcriptase-PCR

Total RNA was extracted using the Thermo Scientific GeneJET RNA Purification Kit (Thermo, Fermentas, USA). Then, 1 μ g of total RNA was converted into the complementary deoxyribonucleic acid (cDNA) using Aid First Strand cDNA Synthesis Kit (Thermo, Fermentas, USA) as instructed by the manufacturer. The resulting transcripts were then quantified by the real-time quantitative PCR on a StepOne plusTM real-time DNA amplification system (Applied Biosystems, USA) using SYBR[®] Premix Ex TaqTM II kit (Takara, Japan) according to the manufacturer's instructions. Pre-designed primers (QuantiTact Primer Assay; Qiagen, Netherlands) specific for amplification of IL-17, RORC2, IL-23R, and IL-9 were applied. For each sample, transcript quantity was normalized to the amount of beta-actin expression.

Table 1: Clinical characteristics of the CVID patients and control subjects

Patient's number	Sex	Diagnosed/year	IgG (g/l)	IgM (g/l)	IgA (g/l)	Volunteer's number	Sex	IgG (g/l)	IgM (g/l)	IgA (g/l)
P1	Female	3	0.75	0.05	0.00	1	Female	5.5	0.6	0.9
P2	Male	18	2.87	0.33	0.10	2	Male	7.8	1.2	0.6
P3	Male	5	3.60	0.25	0.25	3	Male	6.7	1.5	1.4
P4	Female	5	1.23	0.28	0.09	4	Female	9.5	1.0	1.0
P5	Female	19	0.30	0.20	0.00	5	Female	7.6	0.7	1.9
P6	Male	1	2.52	0.33	0.13	6	Male	6.1	1.3	0.9
P7	Male	39	0.18	0.08	0.02	7	Male	9.9	0.8	0.5
P8	Male	10	0.20	0.10	0.00	8	Male	7.6	1.6	1.4
P9	Female	2	0.07	0.25	0.10	9	Female	10.1	0.9	0.7
P10	Female	15	0.40	0.22	0.07	10	Female	6.4	1.1	0.8
Normal range	-	-	4-11	0.5-1.8	0.1-2.0	-	-	4-11	0.5-1.8	0.1-2.0

CVID = Common variable immunodeficiency

Amplification was carried out in a total volume of 20 μ l for 40 cycles of 30 s at 95°C and 30 s at 60°C.

Cell staining and flow cytometric analysis

Whole blood samples with the ethylenediaminetetraacetic acid were collected and lysed using a red blood cell lysis solution (CMG, Iran) according to the manufacturer's instructions. Staining for flow cytometry was performed using the fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-CD19 (eBiosciences, USA), and anti-CD56 antibody (NCAM) (Santacruz Biotech, USA), all for lineage exclusion; peridinin chlorophyll protein (PerCp)-conjugated anti-human CD127 (IL-7R α) antibody (R&D Systems, USA) and phycoerythrin (PE)-conjugated anti-human CD90 (Thy-1) antibody (eBiosciences, USA) for detecting ILCs.^[17] A minimum of 100,000 lymphocytes were acquired per sample. Prepared samples were acquired on a FACScan and data were analyzed with the CellQuest-Pro software (BD Biosciences, San Jose, CA, USA). In order to further investigate the relationship between the IL-17 producing cells and the CVID patients, we counted IL-17 producing ILCs, represented by Lin⁻/CD127⁺/CD90⁺ phenotype, in the peripheral blood of the cases and controls using the flow cytometry method. At the first, in order to exclude the T-cells, B-cells, and natural killer (NK) cells which express CD3, CD19 and CD56, respectively, we analyzed the cells for the absence of these markers (e.g., Lin⁻). For this analysis, we depleted the whole peripheral blood cell samples of most T-cells (with anti-CD3), B-cells (with anti-CD19), and NK cells (with anti-CD56). After gating on Lin⁻ cells, a population of CD127⁺/CD90⁺ cells distinct from the conventional lymphoid cells was present [Figure 1].

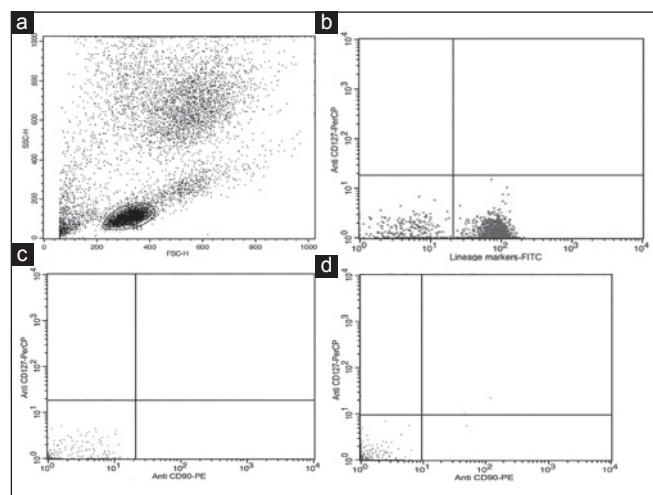


Figure 1: Flow cytometry analysis of the Lin⁻/CD127⁺/CD90⁺ innate lymphoid cells in the peripheral blood of the Common variable immunodeficiency (CVID) patients compared with the healthy individuals. (a) FSC/SSC diagram of peripheral blood cells shows gated area for lymphoid cells. (b) Flow cytometry analysis of the peripheral blood samples in which T cells, B-cells and NK cells are detected as one population using the FITC-conjugated anti-CD3, anti-CD19, and anti-CD56 antibodies. (c) Percentage of the Lin⁻/CD127⁺/CD90⁺ ILCs in a CVID patient. No ILCs was found in the studied patients. (d) Percentage of the Lin⁻/CD127⁺/CD90⁺ ILCs in a normal control

Thus, the Lin⁻/CD127⁺/CD90⁺ cells most likely represented an ILC population.

Statistical analysis

The results were analyzed statistically with the independent sample *t*-test using SPSS 16.0 software (Chicago, USA). Results are expressed as mean \pm standard error of mean, and $P < 0.05$ were considered to be significant.

RESULTS

Table 1 summarizes the clinical characteristics of the CVID patients and control subjects. Three out of 10 CVID patients were suffering from the autoimmune diseases. Our results displayed that relative expression of IL-17 (1.22 ± 0.22 vs. 2.21 ± 0.95) and RORC2 (1.0 ± 0.65 vs. 3.01 ± 2.06) genes in the CVID patients were significantly less than the controls ($P = 0.049$ and $P = 0.046$, respectively). However, this difference for the IL-23R mRNA level was insignificant ($P = 0.99 \pm 0.29$ vs. 1.23 ± 0.44 , $P = 0.252$). Reversely, we observed that gene expression level of the IL-9 in the CVID patients was increased significantly compared with the controls (9.67 ± 0.27 vs. 1.54 ± 0.88 , $P = 0.001$) [Figure 2]. Table 2 shows the detailed information of the cytokines mRNA levels in the patients and normal groups. Our results showed that the number of Lin⁻/CD127⁺/CD90⁺ ILCs were significantly lower in the peripheral blood of the CVID patients compared with the healthy individuals (0.0% vs. $0.036\% \pm 0.02$, respectively, $P = 0.04$) [Figure 1].

DISCUSSION

The present study is aimed to measure the Th17 cell specific gene expression, to measure the mRNA level of IL-9, and

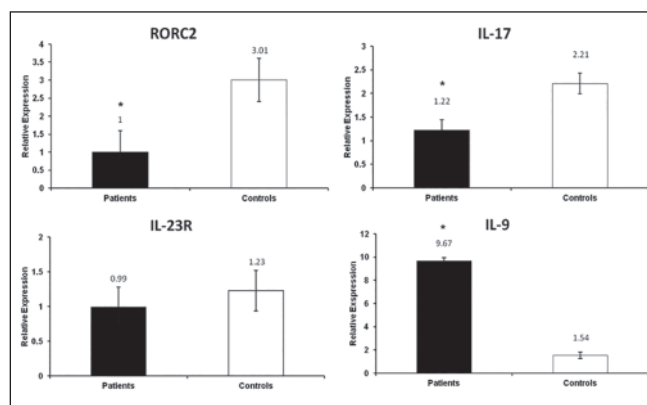


Figure 2: Comparison of the gene expression of IL-17, RORC2, IL-23R, and IL-9 in the peripheral blood of the CVID patients with the healthy individuals by the quantitative reverse transcriptase-polymerase chain reaction. Black bars represent gene expression of the patients and white bars show gene expression of the healthy individuals. The findings showed a decrease in transcript levels of IL-17, RORC2, and IL-23R ($P = 0.049$, $P = 0.046$, and $P = 0.252$, respectively) and strongly increased in transcript levels of IL-9 ($P = 0.001$)

Table 2: Transcript levels of IL-17, RORC2, IL-23R and IL-9 in the control subjects and patients

Volunteers	IL-17 gene expression	RORC2 gene expression	IL-23R gene expression	IL-9 gene expression	Patients	IL-17 gene expression	RORC2 gene expression	IL-23R gene expression	IL-9 gene expression	OID
1	1.75	1.56	0.76	0.39	P1	1.28	0.27	0.57	0.47	No
2	4.34	6.03	0.90	1.96	P2	0.52	0.65	0.24	4.09	Autoimmune hepatitis
3	2.07	4.89	0.92	1.58	P3	0.91	0.65	1.04	1.86	No
4	2.05	3.42	1.20	2.31	P4	1.15	2.45	0.93	15.52	No
5	1.53	2.36	1.13	1.89	P5	1.54	0.92	1.76	24.87	Autoimmune thrombocytopenia
6	3.01	2.99	0.95	3.25	P6	2.80	1.02	0.98	8.52	Respiratory and ocular allergy
7	2.68	3.68	1.21	0.87	P7	3.36	1.43	2.83	26.47	Chronic arthritis
8	1.08	1.40	2.13	1.16	P8	0.46	0.25	0.50	7.37	Respiratory allergy
9	1.35	1.68	1.85	0.42	P9	0.03	0.98	0.61	2.53	No
10	2.19	2.11	1.21	1.55	P10	0.12	1.40	0.39	4.96	Respiratory allergy
Average	2.21	3.01	1.23	1.54	Average	1.22	1.0	0.99	9.67	-
SEM	0.95	2.06	0.44	0.88	SEM	0.22	0.65	0.29	0.27	-

OID = Other immunologic disorders; IL = Interleukin; RORC2 = Retinoic acid receptor-related orphan receptor C2; SEM = Standard error of mean

to count the ILCs in the peripheral blood of the CVID patients. The frequencies of IL-17 producing cells in the CVID patients was markedly decreased compared with the healthy individuals. These cells are known to be involved in inducing inflammation and autoimmunity.^[23] On the other hand, IL-17 alone promotes the human B-cell survival and synergizes with the B-cell activating factor to induce the B-cell proliferation and differentiation into the antibody secreting plasma cells.^[24] Accordingly, it is shown that the Th17 cells act as B-cell helpers.^[25]

Our results showed that the transcript levels of IL-17 in the CVID patients were decreased significantly. Barbosa *et al.* (2011) have shown decreased frequency of IL-17 producing CD4⁺ T in CVID patients compared with the healthy individuals.^[13] IL-17 is also produced by CXCR5⁺ CD4⁺ (T_{FH}) cells and according to a study, the circulating CXCR5⁺ CD4⁺ T-cell (T_{FH}) population were significantly diminished in the CVID patients compared with the healthy subjects.^[26,27] Therefore, this decrease of IL-17 in the CVID patients may be attributable to their diminished T_{FH} population. Our findings are consistent with the results of these studies.

As RORC2 is the key transcription factor for Th17 phenotype it seems that this factor, along with IL-17, can be a specific marker, which helps to better characterizing Th17 cells.^[7,9] Our findings showed a significant decrease in the transcript level of RORC2 in the CVID patients. This is consistent with the diminished frequency of Th17 cells in the CVID patients reported by Barbosa *et al.*^[13] This finding also agreed with the corresponding diseases such as, ICOS-deficient and hyper-IgE patients.^[28,29]

We also showed that the transcript levels of IL-23R, another Th17 characteristic gene decreased slightly or remained unchanged in patients with CVID. This slight decrease may be due to the expression of IL-23R on other cells such as NK cells, dendritic cells and memory T-cells.^[30] We did not find any report in this context in order to compare with our findings. However, this finding is consistent with the diminished Th17 cell count in the Barbosa's report and reduced RORC2 and IL-17 mRNA levels as other Th17 characteristic genes in the present study.

On an average 70-80% of the CVID patients have recurrent sinopulmonary infections, autoimmunity and inflammatory complications. The most common autoimmune conditions are immune thrombocytopenic purpura and hemolytic anemia, but other autoimmune complications arise including RA, pernicious anemia, primary biliary cirrhosis, thyroiditis, sicca syndrome, SLE, and IBD.^[14,31] We next assessed whether these inflammatory and autoimmune manifestations could be related to an expansion of the Th17 cells, as reported in the other autoimmune settings.^[32] There was not any increase in the Th17 cell specific genes expression in the CVID patients with autoimmunity, even when the CVID patients were classified according to the type of the autoimmune manifestation.

Interestingly, we showed that IL-9 mRNA level in the peripheral blood samples of the CVID patients was significantly more than the controls. As shown in Table 2, this increase was too high in two patients with the autoimmune thrombocytopenia (patient 5) and chronic arthritis (patient 7). In addition, in one patient with the autoimmune hepatitis (patient 2), IL-9 gene expression

was increased significantly. IL-17 was diminished strongly in these three patients as well. One study has shown no increase in the level of IL-17 in the CVID patients associated with the Crohn's disease, as compared with the healthy individuals.^[2] This finding is consistent with our results.

According to several studies about the relation of IL-9 and its increase in some of the autoimmune diseases such as SLE, systemic sclerosis, and RA.^[19-21] Our findings suggest that the increase of IL-9 mRNA level may have a role in the pathogenesis of autoimmunity in our CVID patients. However, its exact role and mechanisms need to be confirmed.

Moreover, according to the role of IL-9 in Th17 cells expansion, one can suppose that decreased number of the Th17 cells in the CVID patients may lead to an increase in the IL-9 level as a compensatory effect. On the other hand, in some studies it is demonstrated that IL-9 may exert anti-inflammatory effects in several cells or experimental autoimmune models, presumably through the indirect inhibition of the production of proinflammatory cytokines.^[21] Therefore, increased IL-9 level in the CVID patients might have a casual relation with the IL-17 reduction.

The level of IL-9 transcript was significantly high in one patient without any apparent autoimmunity (patient 4). However, she had two children with the lymphoma disease. It is shown that IL-9 may relate to the Hodgkin lymphoma.^[33] Furthermore, according to its growth/proliferative and anti-apoptotic activities on the different transformed cells, a potential role for this cytokine might be tumorigenesis.^[34] In addition, over-expression of IL-9 induces thymic lymphomas in mice, and IL-9 production is associated with the Hodgkin disease and human T-lymphotropic virus type-1 transformed T-cells in human.^[35] Nevertheless, this patient by herself is not affected by lymphoma.

The mRNA level of IL-9 was also high in three other patients; patient 6 with respiratory and ocular allergy, patients 8 and 10 with respiratory allergy.

The effects of IL-9 have been shown in the development and maintenance of allergic inflammation and airway remodeling. The number of IL-9 mRNA-positive cells in the airway of patients with atopic asthma and allergic rhinitis has shown to be elevated.^[36,37] This is consistent with our findings.

In order to further investigate the IL-17 producing cells, we evaluated the IL-17-producing ILCs population in patients with CVID. These cells are of lymphoid origin^[16] and express IL-7 receptor α -chain (CD127) and Thy-1 (CD90) and are lineage negative.^[17] There is a paucity of information

regarding the ILCs count in different diseases, and this was the first study, which evaluated the ILCs count in the peripheral blood of patients with CVID so far. We showed that the ILCs count in the CVID patients was significantly lower than the controls (0% vs. 0.036% respectively). Average percentage of these cells was 0.036% in the healthy individuals and this is consistent with the study of Mjösberg, who reported this frequency as 0.01-0.03% in the normal population.^[16]

CONCLUSIONS

The results presented in this study show that the Th17 cell specific genes expression (as the determinant Th17 cells) and ILCs (another lymphoid source of IL-17) are decreased in patients with CVID and this could be an explanation for the defect of their humoral immune response. In addition, elevation of the IL-9 gene expression may shed a new light into the way toward the understanding of the mechanism of autoimmunity in the CVID patients.

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AUTHORS' CONTRIBUTION

All authors have contributed in designing and conducting the study. RY, VH, and MM collected the data and MGH, RSh, and MH did the analysis. All authors have assisted in preparation of the first draft of the manuscript or revising it critically for important intellectual content. All authors have read and approved the content of the manuscript and are accountable for all aspects of the work.

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