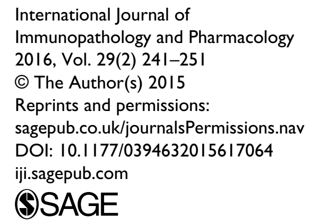

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$CD4^+CD25^+Foxp3^+$ T regulatory cells, Th1 (CCR5, IL-2, IFN-γ) and Th2 (CCR4, IL-4, IL-13) type chemokine receptors and intracellular cytokines in children with common variable immunodeficiency

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Abstract

Common variable immunodeficiency (CVID) is a heterogeneous group of primary antibody deficiencies characterized by decreased serum immunoglobulin G along with a decrease in serum IgA and/or IgM, defective specific antibody production, and recurrent bacterial infections. Abnormal lymphocyte trafficking, dysregulated cellular responses to chemokines, and uncontrolled T cell polarization may be involved in the pathogenesis and may help to understand the clinical complications. We evaluated T helper cell subsets (chemokine receptors CCR4, CCR5, and CCR7), expressions on T lymphocytes, intracellular cytokines – IL-2, IL-4, IL-13, IFN- γ-on $CD4^+$ T cells, and expression of $CD4^+CD25^+Foxp3^+$ regulatory T cells of 20 CVID patients and 26 healthy controls. Autoimmune clinical findings and other complications were also determined. Percentages and absolute numbers of $CD4^+CD25^+$ $Foxp3^+$ cells did not show any significant difference between CVID cases and healthy controls nor between severe and moderate disease patients. The only significant difference regarding Th1 and Th2 type intracellular cytokines was the decreased absolute numbers of $CD3^+CD4^+IL4^+$ cells in CVID cases. There were some findings about T helper cell type dominance in CVID patients such as positive correlation between hepatomegaly and high IL-2 and IFN-γ in $CD3^+CD4^+$ cells and very high expression of CCR5 (Th1) on $CD3^+CD4^+$ cells in patients with granuloma. Th1 (CCR5) and Th2 (CCR4) type chemokine receptors did not show any dominance in CVID cases. However, frequencies of CCR7 expressing $CD3^+$ T cells, $CD3^+CD4^+$ T helper cells and $CD3^+CD8^+$ T cytotoxic cells were significantly lower in severe CVID patients. In addition, presence of autoimmune clinical findings was negatively correlated with $CCR7^+$ cells. As CCR7 is a key mediator balancing immunity and tolerance in the immune system, the abnormality of this mediator may contribute to the profound immune dysregulation seen in CVID. In addition, Th1 cells seem to be more involved in the disease pathogenesis than Th2 cells.

Keywords

CVID, intracellular cytokines, Th1/Th2 chemokine receptors, Treg cells

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Introduction

Common variable immunodeficiency (CVID) is a heterogeneous group of primary antibody deficiencies characterized by decreased serum immunoglobulin (Ig) G along with a decrease in serum IgA

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and/or IgM, defective specific antibody production, and recurrent bacterial infections.[1] Most patients present with recurrent upper and lower respiratory tract infections. Various autoimmune conditions, particularly autoimmune thrombocytopenia and autoimmune hemolytic anemia, are common in CVID, occurring in approximately 20% of patients.[2–4] Underlying causes of autoimmunity in CVID are increased tendency towards loss of tolerance and immune dysregulation.[2–4] CVID patients are also prone to develop non-infectious manifestations such as gastrointestinal, lymphoproliferative, and granulomatous complications.[1–3] These complications are more likely to occur in CVID patients with other autoimmune phenomena.[4]

The $CD4^+$ T cells are central regulators of both humoral and cellular immune responses. The subsets of $CD4^+$ T cells, T-helper 1 (Th1), Th2, and regulatory T cells are specialized in regulating different aspects of immunity. Immune balance controlled by Th1 and Th2 cells is critical for the protection of host autoimmune diseases.[5] Th1 cells commonly secrete IL-2 and IFN-γ, activate macrophages, provide helper function for IgM, IgG, and IgA synthesis, and elicit delayed-type hypersensitivity reactions. In contrast, Th2 cells develop in response to allergens or helminth antigens, produce IL-4, IL-5, and IL-13, encourage production of all immunoglobulin classes, including IgE, and suppress cell-mediated immunity.[6,7] The $CD4^+CD25^+$ $Foxp3^+$ T regulatory cells (Treg cells) maintain peripheral tolerance by actively blocking immune responses, inflammation, and tissue destruction by suppressing the functions of an array of cell types including classical $CD4^+$ helper T cells, B-cell antibody production and affinity maturation, $CD8^+$ cytotoxic T lymphocyte granule release, and antigen presenting cell function.[8,9]

Chemokines (chemotactic cytokines) direct lymphocyte trafficking to secondary lymphoid tissues, and facilitate appropriate cellular and humoral interactions which have important implications for disorders of the immune system.[10] On activation, effector lymphocytes upregulate a different set of chemokine receptors on their cell surface, which increase the chemotactic sensitivity of lymphocytes to chemokines. The expression profile of chemokines in a particular inflammation site determines the type of infiltrating leukocytes. The chemokine receptor CCR7 (the receptor for EBV-induced gene 1 ligand [EBI1]) has previously been shown to contribute to the formation of germinal centers and antibody production.[11] Naive T cells express CXCR4 and CCR7. After activation, T cells can be polarized to either Th1 or Th2 type cells. Chemokine receptors CCR5 and CXCR3 are preferentially expressed in Th1 cells over Th2 cells. More CCR3, CCR4 (the receptor for TARC [thymus and activation-regulated chemokine]), and CCR8 are expressed in Th2 cells than Th1 cells.[10–13]

Abnormal lymphocyte trafficking, dysregulated cellular responses to chemokines, and uncontrolled T cell polarization may be involved in the pathogenesis of primary immunodeficiency disorders and may help to understand the clinical complications of these patients. We investigated T helper cell subset profiles of CVID patients and compared these with a group of healthy controls, and tried to find correlations between these levels and clinical complications such as autoimmune diseases or granulomatous lesion formation.

Materials and methods

Twenty patients admitted to Ege University Pediatric Immunology Department were recruited in the study. All patients fulfilled criteria for CVID based on the European Society for Immunodeficiencies / Pan-American Group for Immunodeficiency (ESID/PAGID) definition which stipulates a marked decrease of IgG (of at least two standard deviations [SDs] below the mean for age) and reduced serum IgA and/ or IgM, specific antibody deficiency, age older than 2 years, and exclusion of other causes of hypogammaglobulinemia.[1] Written informed consent was obtained from parents. The study was approved by the local ethics committee.

Clinical information was obtained for each CVID patient from their medical records. Information was collected on gender, date of birth, age at onset of symptoms, age at diagnosis, familial history for primary immunodeficiencies, consanguinity, history of pneumonias, chronic lung disease, bronchiectasis, pulmonary granulomata or lymphoid interstitial pneumonia, severe infections, autoimmune diseases (autoimmune hemolytic anemia [AIHA], autoimmune thrombocytopenia [ITP], rheumatoid arthritis, pernicious anemia), granulomatous infiltration at any localization, presence of splenomegaly, hepatomegaly, malignancy, gluten-like enteropathy, chronic giardiasis, osteoporosis, and splenectomy.

Patients were examined as a whole study group and also they were divided into two subgroups due to previously published disease severity criteria for CVID.[14,15] The severe disease (SD) group included patients who had splenomegaly and/or granulomatous diseases and/or bronchiectasis and/or lower baseline IgG values (lower than 270 mg/dl at admission) (n = 6). The oderate disease (MD) group were CVID patients diagnosed as having ESID/ PAGID criteria but did not fulfill the severe disease group inclusion criteria (n = 14).

All patients were receiving their regular IVIG replacement therapy and none of them had an infectious episode during data analysis.

Whole blood count assay. Whole blood count; leukocyte counts, absolute neutrophil, and lymphocyte counts were performed with hemocounter (Cell-Dyn 3700, Abbott Diagnostics, USA).

Serum immunoglobulin assay. The quantifications of serum IgG, IgM, and IgA had been performed by nephelometric method with Dade Behring BNII Nephelometer Analyzer and commercially available kits by Dade Behring (Germany) and were investigated in comparison to Turkish age-related normal levels.[16]

Flow cytometric analyses. All flow cytometric analyses were made by using FacsCalibur (Becton Dickinson, USA). The instrument has two lasers for excitation; 488 nm (primary) can detect scattered light in the form of forward scattered light FSC (for cell size), side scattered light SSC (for cell complexity/granularity), FL-1 (FITC), FL-2 (PE), FL-3 (PERCP, PERCPCY5.5), and 635 nm (secondary) can detect FL-4 (APC). Emission filters for FITC, PE, PERCP, and APC are as 530/30, 585/42, 670LP, and 661/16, respectively. The flow cytometric data were given in the text according to standardized publishing rules.[17–19]

Lymphocyte subsets assay

The percentages and absolute counts of lymphocyte subsets ($CD3^+$ T cells, $CD19^+$ B cells, $CD3^+CD4^+$ T helper cells, $CD3^+CD8^+$ T cytotoxic cells, $CD3^+HLA\text{-}DR^+$ activated T cells, $CD3^-CD16^+CD56^+$ natural killer cells, $CD19^+CD27^+$ memory B cells, $CD19^+CD27^+IgD^-IgM^-$ switched memory B cells) were investigated by flow cytometry (FACSCalibur, Becton Dickinson, USA) using peripheral blood anti-coagulated with EDTA. CD3/CD4/CD8/CD45, CD3/CD19/CD16/56/CD45, CD3/HLA DR multicolor antibody reagents, and anti-CD27 FITC (clone M-T271), anti-IgD PE (clone IAG2), and anti-IgM PE (clone G20-127) were all purchased from BD Biosciences. Gating strategy for defining the memory and switched memory B cells was as follows: $CD19^+$ B lymphocytes were gated on their forward and side scatters. All cell subpopulations were acquired using Cell Quest-Pro software (BD Biosciences, Belgium) and compensations were made.

Chemokine receptor (CCR4, CCR5, and CCR7) expressions on T lymphocytes

The T cell compartments were analyzed with four-color FACSCalibur flow cytometry (Becton-Dickinson, USA) using peripheral blood anti-coagulated with heparin. The chemokines studied on lymphocytes were as follows; CCR4 for Th2, CCR5 for Th1, and CCR7 for naive T cells. Cells were stained with anti-CD3-APC (clone SK7), anti-CD4-PerCP (clone SK3), anti-CD194-PE (CCR4) (clone 1g1), anti-CD195-FITC (CCR5) (clone 2D7/CCR5), and anti-CD197-PE (CCR7) (clone JD12). All monoclonal antibodies were obtained from Becton-Dickinson, USA. Gating strategy for defining the chemokine receptors expressions was as follows: $CD3^+$ T lymphocytes were gated based on their forward and side scatters. Compensations were made if needed and the percentages of the chemokines were assessed from $CD3^+CD4^+$ lymphocytes using Cell-Quest software (Becton Dickinson, USA).

Intracellular cytokine expressions (IL-2, IL-4, IL-13, IFN- γ) on CD4+ T cells

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque gradient centrifugation (Biochrome). These PBMC were stained for surface and intracellular markers. IL-2 (PE, clone5344-111) and IFN-γ (PE, clone 25723.11) were the intracellular Th1 cytokines, IL-4 (PE, clone 3010.211) and IL-13 (PE, clone JES10-SA2) were the intracellular Th2 cytokines studied on lymphocytes. All monoclonal antibodies were derived from Becton Dickinson, USA. Lymphocytes were activated by phorbol 12-myristate 13 acetate (PMA)

Table 1. Percentages of intracellular cytokines (IL-2, IL-4, IL-13, and IFN-γ) before and after stimulation in CVID patients and control groups.

		Before stimulation	After stimulation	*P*
CD3+CD4+IL2+ %	Control group	0.10 ± 0.09	6.5 ± 11.6	0.046
	Patient group	0.02 ± 0.02	11.7 ± 9.5	0.000
CD3+CD4+IL4+ %	Control group	1.35 ± 1.37	3.19 ± 2.76	0.030
	Patient group	0.26 ± 0.34	1.91 ± 2.11	0.003
CD3+CD4+IL13+ %	Control group	0.13 ± 0.19	0.64 ± 0.76	0.025
	Patient group	0.06 ± 0.12	0.77 ± 0.63	0.001
CD3+CD4+IFNγ+ %	Control group	1.03 ± 1.15	6.93 ± 8.00	0.009
	Patient group	0.33 ± 0.41	8.66 ± 7.86	0.001

(Sigma-Aldrich, USA) + ionomycine (I) (Sigma Aldrich, USA) permeabilizing solution (Becton Dickinson, Belgium), and brefeldin-A (BFA, Sigma Aldrich, USA) and anti-CD69 PE (clone L78) monoclonal antibody were used in accordance with manufacturer's recommended protocol for the detection of intracellular cytokines in activated lymphocytes. Gating strategy for defining the intracellular cytokine expressions in patients with altered percentages compared to representative healthy controls was as follows: lymphocytes were gated based on their forward and side scatters. The percentages of the IL-2 and IFN-γ in $CD4^+$ T cells gate were assessed to determine intracellular Th1 type cytokine expression. The percentages of Th2 type cytokine expressions (IL-4 and IL-13) were determined in $CD4^+$ T cells, also. All samples were analyzed by flow cytometer (FACSCalibur, Becton-Dickinson, USA) using Cell-Quest software (Becton-Dickinson, USA) and also compensation was made if it was needed. The percentages of intracellular cytokines of $CD4^+$ T lymphocytes obtained before and after activation were compared for both study and control groups (Table 1) and all intracellular cytokine detections were found to be significantly increased, showing that lymphocytes were activated properly.

Expression of Treg cells

For the intracellular detection of Foxp3, heparinized blood was first stained with anti-CD25-FITC (clone 2A3) and anti-CD4-APC (clone RPA-T4) (BD Pharmingen) for cell surface markers, and then cells were washed in PBS containing FBS and 0.1% sodium azide for cell surface markers. After washing, cells were fixed and permeabilized (permeabilizing buffer, cat. No. 560098) with the appropriate buffers and then stained with intracellular PE-conjugated anti-Foxp3 (clone 259 D/C7) antibody (BD Pharmingen) according to the manufacturer's protocol. Gating strategy for defining the Treg cells' frequency in patients with altered percentages compared to representative healthy controls was as follows: $CD4^+$ lymphocytes were gated based on their forward and side scatters. Compensation and the percentage of stained Treg cells was measured by calculating the percentage of $CD25^+$ $FoxP3^+$ double-positive cells within $CD4^+$ population using Cell-Quest software (Becton Dickinson, Belgium) and Flow-Jo software version 8.8.9 (Treestar, US). For determination of Treg frequencies, $CD4^+$ cells with low side scatter were identified and then a forward vs. side scatter gate applied to isolate small lymphocytes and to facilitate the exclusion of cellular debris and non-viable cells. Positive fluorescence staining was established using isotype controls.

Evaluation of growth. Height and weight SDS of patients were determined according to Turkish children's standards.[20] Pubertal development was evaluated according to the method of Tanner and bone age.[21,22] A growth rate below the appropriate growth velocity for age was regarded as growth failure.

Evaluation of autoimmunity. In order to evaluate autoimmunity in patients, anti-nuclear antibody (ANA) positivity in serum was determined by immunoflorescence (IF) on mosaic Hep-20-10/liver monkey cell slides (Euroimmun, Lübeck, Germany) in a double-blind setting. ANA IF titers of 1/100 were taken as cutoff titers. Anti-neutrophil cytoplasmic antibody (ANCA) positivity with a 1/16 cutoff titer was also evaluated by immunoflorescence. Titrimetric qualitative determinations (+/–) were used. All autoimmune diseases (autoimmune hemolytic anemia, autoimmune thrombocytopenia, rheumatoid arthritis, pernicious anemia) records were also evaluated.

Evaluation of coeliac-like disease findings. Gluten-like enteropathy was evaluated from patients' data records for anti-gliadin (AGA) and anti-tissue transglutaminase (TTG) IgA antibodies levels which were performed with ELISA. The clinical and also endoscopic findings which were supported with gastroenterology consults confirmed the gluten-like enteropathy diagnosis.

Evaluation of osteoporosis. Bone mineral density (BMD) of the lumbar spine and femoral neck was measured with dual energy X-ray absorptiometry (DEXA, Hologic QDR 4500A, Waltham, MA, USA) (g/cm^2). The results of BMD were compared to healthy age- and sex-matched Turkish children and *Z*-scores were calculated.[16]

All data were expressed as mean plus or minus SD except when indicated otherwise. SPSS 17 program was used for statistical analysis. Correlation comparisons between paired samples were made by Pearson's product moment correlation coefficient. Statistical comparisons of numeric data were made using Student's t-test. The classified data were evaluated by χ^2 test. Mann-Whitney U test was used for comparisons between clinical parameters and variables. Differences between groups were considered significant at $P < 0.05$.

Results

A total of 20 patients (17 boys, 3 girls) and 26 age-matched controls (12 boys, 14 girls) were included. Mean age of the study group, age at the beginning of symptoms, and mean age at diagnosis were 173.3 ± 77.9 months, 70.1 ± 60.8 months, and 93.3 ± 58.8 months (min. 14 months, max. 204 months), respectively. Mean follow-up time was 80.6 ± 70.3 months. Clinical presentations were as follows: recurrent infections (n = 18, 90%), vasculitis (n = 1, 5%), and autoimmune cytopenia (hemolytic anemia and thrombocytopenia) (n = 1, 5%). Family history for primary immune deficiencies (consanguinity, primary immunodeficiency history in brothers/sisters/first-degree relatives) was positive in five patients (25%). Fifteen percent of the patients (n = 3) were born to consanguineous parents.

The major complications observed during follow-up were as follows: bronchiectasis (n = 4, 20%), splenomegaly (n = 5, 25%), osteoporosis (n = 6, 30%), delayed growth (n = 6, 30%), autoimmunity (n = 5, 25%), hepatic enlargement (n = 5, 25%), gluten-like enteropathy (n = 2, 10%), granuloma formation (n = 3, 15%), and lymphoma (n = 2, 10%). Autoimmune clinical findings were observed in five patients: chronic arthritis (n = 1), inflammatory bowel disease (n = 2), relapsing polychondritis (n = 1), spondylarthropathy of the hip (n = 1), sacroileitis (n = 1), autoimmune hemolytic anemia (n = 1), thrombocytopenia (n = 2), and vasculitis (n = 1). Growth failure, autoimmunity, gluten-like enteropathy, osteoporosis, bronchiectasis, splenomegaly, and hepatomegaly were the complications which were significantly prevalent in the severe disease group (Table 2). The complications that presented on admission and developed during follow-up and as well as their observation ages are listed in Table 3.

At the time of diagnosis, before starting intravenous immunoglobulin (IVIg) therapy, baseline IgG level in the whole study group was 405.8 ± 181.6 mg/dl, IgM was 43.4 ± 60.3 mg/dl, and IgA was 43.3 ± 41.6 mg/dl, respectively (the normal immunoglobulin reference range for the mean age of study group '173 months': IgG: 794.4–1209.8, IgM: 61.5–161, IgA: 84.6–143.4 mg/dl). The percentages of $CD3^+CD8^+$ T cytotoxic cells and $CD3^+$HLA-DR^+ T cells were significantly higher, $CD19^+$ B cells were lower in study group compared to controls (Table 4). White blood cell (WBC) counts, absolute lymphocyte counts (ALC), $CD3^+$ T cells, $CD19^+$ B cells, $CD19^+CD27^+$ memory B cells, $CD3^+CD4^+$ T helper cells, $CD3^+CD8^+$ T cytotoxic cells, $CD3^-CD16^+CD56^+$ natural killer cells, and HLA-DR^+ active T cells of the severe and moderate disease groups did not show any statistically significant difference (Table 4). The percentage of class-switched memory B cells ($CD19^+CD27^+IgM^+IgD^-$) was significantly lower in severe patients, although the absolute numbers did not differ. All patients were given regular IVIG replacement therapy with a mean interval of 42.0 ± 11.8, 27.7 ± 4.5, and 50.0 ± 7.7 days in the whole, SD, and MD groups, respectively ($P = 0.001$).

The percentages and absolute counts of Treg cells, chemokine receptor expressing T cells, and intracellular IL-2, IFN-γ, IL-4, and IL-13 expressing T helper cells are listed in Table 4. Neither patient and control groups nor severe disease and moderate groups showed any significant difference in terms of percentages and absolute counts of Treg cells, CCR4 (Th2), and CCR5 (Th1) expressing T helper cells. The percentages of CCR7 expressing $CD3^+$ total T cells ($P = 0.001$), $CD3^+CD4^+$ T helper

Table 2. The complications that have been observed during follow-up in CVID patients (*P <0.05; **Z scores of severe disease group: mean: –2.37 ± –0.38; min_–max: –2.1_–3.1).

	Whole group (n = 20)		Severe disease group (n = 6)		Moderate disease group (n = 14)		P
	n	%	n	%	n	%	
Growth failure	6	30	6	100	–	–	0.000*
Splenomegaly	5	25	4	66.7	1	7.1	0.006*
Hepatomegaly	5	25	4	66.7	1	7.1	0.006*
Bronchiectasis	4	20	3	50	1	7.1	0.032*
Autoimmunity	5	25	4	66.7	1	7.1	0.006*
Giardiasis	3	15	2	33.3	1	7.1	0.143
Osteoporosis	6	30	6**	100	–	–	0.000*
Gluten-like enteropathy	2	10	2	33.3	–	–	0.026*
Granuloma formation	3	15	2	33.3	1	7.1	0.143
Lymphoma	2	10	1	16.7	1	7.1	0.526

Table 3. The complications that presented on admission and developed during follow-up as well as their observation ages.

Patient no. with complications	Age (months)	Age on admission (months)	Complications present on admission	Complications developed during follow-up/age (months)
1	206	143	Osteoporosis Granuloma formation (lungs) Growth failure	Lymphoma (204)
2	147	36	(–)	Bronchiectasis (72) Osteoporosis (96) Granuloma formation (liver) (100)
3	209	193	Osteoporosis Growth failure	(–)
4	204	180	Lymphoma (remission)	(–)
5	169	134	Osteoporosis Growth failure	Bronchiectasis (161)
6	350	96	Gluten-like enteropathy Growth failure Bronchiectasis	Osteoporosis (148)
7	276	144	Gluten-like enteropathy Growth failure	Bronchiectasis (152) Osteoporosis (156)
8	128	96	Bronchiectasis	(–)
9	132	42	(–)	Granuloma formation (lung) (84)

cells ($P = 0.038$), and $CD3^+CD8^+$ T cytotoxic cells ($P = 0.038$) were significantly lower in patients with severe complications, whereas absolute numbers of these cells did not show any statistically significant difference ($P > 0.05$) (Table 5). There was no significant correlation between Treg cell levels and autoimmune findings ($P = 0.419$, r = −0.203) (Table 6). On the other hand, presence of autoimmune clinical findings was negatively correlated with CCR7 expression on naive $CD3^+$, $CD4^+$, and $CD8^+$ T cells that means as $CCR7^+$ T cells decrease, autoimmune findings are more observed (Table 6).

Intracellular IL-2, IL-13, and IFN-γ expressions did not differ in CVID patients and control group in terms of frequency and absolute numbers. Absolute number of $CD3^+CD4^+IL4^+$ cells were found to be significantly lower in whole disease group compared to healthy controls (8.7 ± 10.2 vs. 42.2 ± 55.7) ($P = 0.032$) (Table 5).

Patients with hepatomegaly had higher IL-2 and IFN-γ on stimulated $CD3^+CD4^+$ cells (Table 6).

Table 4. Immunologic findings in CVID patients and control groups (*P <0.05).

		Control group (n = 26)	Patient group (n = 20)	*P*	Severe disease group (n = 6)	Moderate disease group (n = 14)	*P*
WBC /mm^3		7214.3 ± 2288.1	7470.1 ± 4395.6	0.826	8833.3 ± 7153.0	6789.2±2254.8	0.522
ALC /mm^3		2701.9 ± 1054.5	2524.1 ± 1706.3	0.704	2858.7 ± 2454.7	2358.3±1290.0	0.658
CD3$^+$	Cells /mm^3	1918.3 ± 240.4	1968.7 ± 282.6	0.278	2112.5 ±428.8	1879.5 ±219.3	0.287
	%	71.1 ± 8.9	78.0 ± 11.2	0.076	73.9 ± 15.0	79.7±9.3	0.413
CD19$^+$	Cells /mm^3	351.2 ± 143.2	199.4 ± 161.5	0.871	214.4 ±277.2	179.2 ±115.5	0.507
	%	13.0 ± 5.3	7.9 ± 6.4	**0.005***	7.5 ± 9.7	7.6 ± 4.9	0.981
C19$^+$CD27$^+$	Cells /mm^3	68.4 ± 26.7	56.3 ± 97.2	0.765	21.9 ± 14.3	18.1 ± 6.5	0.262
	%	19.9 ± 8.7	19.3 ± 9.5	0.972	14.3 ± 5.8	6.5±1.8	0.441
CD19$^+$CD27$^+$IgM$^-$IgD$^-$	Cells /mm^3	40.5 ± 33.0	54.8 ± 13.5	0.697	20.0 ± 16.8	37.0 ± 32.9	0.378
	%	19.0 ± 11. 4	21.4 ± 16.9	0.283	5.1 ± 7.1	29.6 ± 13.9	**0.001***
CD3$^+$CD4$^+$	Cells /mm^3	761.5 ± 174.5	736.2 ± 257.8	0.694	735.1 ± 401.3	723.6 ± 176.6	0.517
	%	39.7 ± 9.1	37.4 ± 13.1	0.495	34.8 ± 19.0	38.5 ± 9.4	0.664
CD3$^+$CD8$^+$	Cells /mm^3	502.5 ± 118.9	700.8 ± 257.8	0.143	747.8 ± 266.1	670.9 ± 257.4	0.173
	%	26.2 ± 6.2	35.6 ± 13.1	**0.007***	35.4 ± 12.6	35.7 ± 13.7	0.968
CD3$^-$CD16$^+$56$^+$	Cells /mm^3	324.2 ± 148.6	229.6 ± 209.5	0.187	311.5 ± 365.9	301.8 ± 136.7	0.781
	%	12.1 ± 5.5	9.1 ± 8.3	0.172	10.9 ± 12.8	12.8 ± 5.8	0.646
CD3$^+$HLA DR$^+$	Cells /mm^3	117.0 ± 59.4	352.3 ± 354.3	**0.006***	509.1 ± 234.4	287.2 ± 375.9	0.754
	%	6.1 ± 3.1	17.9 ± 18.0	**0.009***	24.1 ± 11.1	15.2 ± 20.0	0.222

Table 5. The percentages and absolute counts of Treg cells, chemokine receptors (CCR4 [CD194], CCR5 [CD195], CCR7 [CD197]), and intracellular cytokines (IL-2, IL-4, IL-13, and IFN-γ) in CVID patient and control groups (*P <0.05, †levels on stimulated lymphocytes).

		Control group (n = 26)	Whole group (n = 20)	*P*	Severe disease group (n = 6)	Moderate disease group (n = 14)	*P*
CD4$^+$CD25$^+$Foxp3$^+$	%	1.88 ± 1.96	3.52 ± 2.30	0.098	3.24 ± 2.99	3.66 ± 1.22	0.763
	Cells /mm^3	19.7 ± 24.3	29.1 ± 39.5	0.571	31.9 ± 62.8	27.2 ± 15.8	0.831
CD3$^+$CD4$^+$CD194$^+$	%	21.2 ± 11.4	16.5 ± 7.81	0.104	19.7 ± 8.63	15.0 ± 7.29	0.277
	Cells /mm^3	230.7 ± 252.8	130.2 ± 142.5	0.226	171.1 ± 218.2	107.9 ± 84.5	0.399
CD3$^+$CD4$^+$CD195$^+$	%	10.4 ± 6.56	10.0 ± 5.01	0.832	13.9 ± 6.58	8.37 ± 3.16	0.095
	Cells /mm^3	80.8 ± 69.1	70.6 ± 66.7	0.739	92.1 ± 108.4	58.9 ± 28.5	0.342
CD3$^+$CD4$^+$CD197$^+$	%	28.7 ± 12.7	29.4 ± 15.4	0.860	18.8 ± 12.5	34.0 ± 14.5	**0.038***
	Cells /mm^3	335.0 ± 227.7	247.6 ± 237.4	0.416	176.6 ± 261.8	286.3 ± 226.3	0.380
CD3$^+$CD8$^+$CD197$^+$	%	10.9 ± 5.91	10.9 ± 7.27	0.999	4.92 ± 2.94	13.5 ± 7.06	**0.038***
	Cells /mm^3	73.1 ± 57.1	96.7 ± 127.1	0.654	101.8 ± 203.2	65.4 ± 71.9	0.924
CD3$^+$CD197$^+$	%	36.4 ± 16.8	40.1 ± 21.5	0.533	24.5 ± 14.9	46.8 ± 20.7	**0.001***
	Cells /mm^3	1137.7 ± 641.1	812.6 ± 664.4	0.283	502.1 ± 670.6	981.9 ± 626.2	0.161
CD3$^+$CD4$^+$IL2$^{+\dagger}$	%	6.56 ± 11.6	11.7 ± 9.50	0.173	16.6 ± 11.6	9.13 ± 7.46	0.198
	Cells /mm^3	23.8 ± 25.6	60.7 ± 45.3	0.104	74.8 ± 44.9	51.3 ± 45.6	0.344
CD3$^+$CD4$^+$IL4$^{+\dagger}$	%	3.19 ± 2.76	1.91 ± 2.11	0.151	1.48 ± 1.09	2.15 ± 2.52	0.460
	Cells /mm^3	42.2 ± 55.7	8.7 ± 10.2	**0.032***	4.8 ± 3.1	11.3 ± 12.6	0.242
CD3$^+$CD4$^+$IL13$^{+\dagger}$	%	0.64 ± 0.76	0.77 ± 0.63	0.591	1.10 ± 0.61	0.59 ± 0.59	0.129
	Cells /mm^3	2.8 ± 1.6	3.6 ± 3.1	0.562	4.2 ± 3.1	3.2 ± 3.1	0.556
CD3$^+$CD4$^+$ IFN$\gamma^{+\dagger}$	%	6.93 ± 8.00	8.66 ± 7.86	0.536	12.23 ± 7.37	6.72 ± 7.74	0.176
	Cells /mm^3	44.4 ± 39.7	78.2 ± 57.1	0.157	53.6 ± 41.1	38.2 ± 40.1	0.484

Patients with granuloma were found to have higher CCR5 expression on CD3$^+$CD4$^+$ cells ($P = 0.002$, r = 0.656) suggesting that Th1 cells may play a role in granuloma formation (Table 6).

Discussion

CVID is highly heterogeneous immunodeficiency syndrome and may present at any age. About 20% of patients have a family history of

Table 6. Statistically significant correlations between chemokine receptor profiles, intracellular cytokine expressions, and complications and clinical findings observed at admission and follow-up.

		P	r
Hepatomegaly	$CD3^+CD4^+IL\text{-}2^+$	0.026	0.536
	$CD3^+CD4^+IFN\text{-}\gamma^+$	0.001	0.734
Granuloma	$CD3^+CD4^+CD195^+$	0.002	0.656
Autoimmunity	$CD3^+CD197^+$	0.009	−0.567
	$CD3^+CD4^+CD197^+$	0.006	−0.587
	$CD3^+CD8^+CD197^+$	0.016	−0.530

immune deficiency and parental consanguinity is a well-known risk factor. In our study, mean age at diagnosis was about 8 years, and family history and parental consanguinity were 25% and 15%, respectively. Although diagnostic delay was not so long (about 2 years), 20% of patients were presenting bronchiectasis and 30% were presenting osteoporosis and growth failure.

Some CVID patients may present low peripheral B cell numbers associated with defective terminal B cell maturation. Kreuzaler et al. reported that 10% of CVID patients have low B cell counts.[23] Similar to our previous study,[15] there was no significant difference between severe and moderate patient groups in terms of B cell numbers; however, B cells in total CVID patients were significantly lower than healthy controls (Table 4). Previously, it has been reported that there is a decrease in switched memory B cells in CVID patients which is more severe in patients with additional lymphoproliferative, autoimmune, and digestive complications.[15] In our study, similar to all other studies, $CD19^+CD27^+$ IgM^+IgD^- switched memory B cells were found to be significantly decreased in severe CVID patients.

Autoimmune disorders occur with a higher incidence in CVID patients than in the general population. Approximately 20–30% of CVID patients develop autoimmune disorders which sometimes present as the first manifestation of the disease.[23] Juvenile idiopathic arthritis (JIA), pernicious anemia, autoimmune tiroiditis, alopecia areata, primary biliary cirrhosis, vitiligo, and systemic lupus erythematosus have been described in CVID, however the most common types reported are idiopathic thrombocytopenic purpura (ITP) and autoimmune hemolytic anemia (AIHA).[23] AIHA (4/14, 28.5% of total autoimmune diseases observed in the study), JIA (4/14, 28.5%), and ITP (3/14, 21.4%) were the most common autoimmune disorders in Abolhassani et al.'s study.[23] In our study, autoimmune diseases were observed in five cases (25%) and four of them were in the severe disease group (Table 2).

The pathogenesis of autoimmunity in CVID remains obscure. How autoantibodies can be produced against specific tissues in a state of impaired antibody production is unclear.[24] B cells may undergo abnormal somatic hypermutation or there may be a failure to remove self-reactive B cells due to defective receptor editing.[25] The lack of switched memory B cells is a characteristic feature of CVID, especially in the cases with autoimmune diseases.[4] In our study, the proportions of $CD19^+CD27^+IgD^-$ IgM^- switched memory B cells were significantly lower in severe disease group compared to moderate cases ($P = 0.001$) (Table 4) that means patients with splenomegaly, granulomatous diseases, bronchiectasis, and autoimmunity have very low switched memory B cells. On the other hand, there is a growing body of evidence suggesting that an imbalance in the regulatory immune system plays a major role in the pathogenesis of autoimmune disease in CVID patients.[8] A number of genetic and mechanistic defects may lead to defective regulation by Tregs, because of a deficiency in either Treg frequency or function.[8]

Tregs play a central role in maintaining self-tolerance and have been implicated in downregulation of the immune response.[26] Cell-to-cell contact leading Treg-mediated suppression is thought to be the main mechanism. On the other hand, the immunoregulatory role of IL-10 and TGF-β produced by T cells cannot be ruled out.[26] Recently, abnormalities in Treg proportions and functions have been reported in CVID patients.

Genre et al.[27] reported for the first time that CVID patients with autoimmune disease had a significantly reduced frequency of Treg cells in their peripheral blood accompanied by a decreased intensity of Foxp3 expression. Foxp3 expression levels in CVID patients without autoimmunity did not differ from those in healthy controls.[27] In Arumugakani et al.'s study,[28] CVID patients had significantly fewer Treg cells than controls, and low frequency of Treg cells was associated with expansion of $CD21^{lo}$ B cells in patients. In the same study, patients with autoimmunity had significantly reduced frequency but normal numbers of regulatory T cells, while patients with splenomegaly had significant reduction in frequency and number of

regulatory T cells.[28] In Arandi et al.'s first study in Iranian CVID patients, similar results regarding the proportions of Tregs were obtained.[29] In the second study, the levels of immunoregulatory cytokines IL-10 and TGF-β produced by Tregs were also measured. The level of TGF-β did not differ between CVID patients and controls while the amount of IL-10 was remarkably decreased in CVID patients.[26] Melo et al. showed significantly reduced Treg cells in CVID patients ($P = 0.003$) in comparison to healthy subjects.[30] In our study, not only percentages, but also absolute numbers of Treg cells did not show any significant difference between CVID cases and healthy controls and also between severe and moderate disease patients (Table 5). The results of previous studies were contradictory and our data may show that Tregs do not play an important role in the pathogenesis of CVID. The low numbers of patients with autoimmunity may be the reason of this non-significant result, because there was no significant correlation between Treg cell numbers and autoimmune findings observed in our patients (Table 6).

Cytokines play an essential role in antibody synthesis. Th1 cells which synthesize cytokines such as IL-2 and IFN-γ have a role in macrophage activation, whereas Th2 cell cytokines IL-4 and IL-10 have a role in the regulation of the humoral immune response. Although cytokine studies highlighting the role of Th1 and Th2 in CVID T cells has been performed in some studies, the results are contradictory. In Rezaei et al.'s study,[31] Th2 cytokine levels in serum (IL-4 and IL-10) were significantly higher in the CVID group than controls. However, there were no significant differences in Th1 cytokines (IL-2 and IFN-γ) between the two groups.[31] In two other studies by Holm et al.[32] and Zhou et al.,[33] the authors have reported a decreased level of IL-10 in CVID. In our study, the only significant difference regarding Th1 and Th2 type intracellular cytokines is the decreased absolute numbers of $CD3^+CD4^+IL4^+$ cells in CVID cases ($P = 0.032$) (Table 5). There are some other weak evidences about T helper cell type dominance in CVID patients such as positive correlation between hepatomegaly and high IL-2 and IFN-γ in $CD3^+CD4^+$ cells and very high expression of CCR5 (Th1) on $CD3^+CD4^+$ cells in patients with granuloma. Rezaei et al.[31] agreed that formation of granuloma could be associated with a Th1 response. Similar to our study, based on their finding of significantly increased levels of IFN-γ production in T cells, North et al.[34] suggested a Th1 response in CVID, with normal cellular levels of IL-2 production. We suggest that numbers of CVID cases have to be increased in the study group before deciding about Th1 dominance in CVID, because it is a very heterogeneous disease.

The other way to examine Th1/Th2 polarization is to determine chemokine receptors.[34] On activation, lymphocytes upregulate a number of chemokine receptors on their surface, which increases the chemotactic sensitivity of lymphocytes to chemokines.[10] While naive T cells express CCR7 and CXCR4, activated Th1 cells express CCR5 and CXCR3 and Th2 cells express CCR4 and CCR3.[34] Th1 cells bearing CCR5 and CXCR3 are common infiltrates in the rheumatoid synovium whereas Th2 cells bearing CCR3 are predominate in allergic diseases such as asthma and contact dermatitis, but absent from rheumatoid synovium.[10]

There are few studies investigating the role of chemokine receptors in primary antibody deficiencies. Payne et al.[35] hypothesized that abnormal chemokine receptor expression might give rise to defects of lymphocyte migration into lymphoid tissues, and consequently be associated with defective antibody production in primary antibody deficiencies such as CVID and specific polysaccharide antibody deficiency (SPAD). The authors indicated that the expression of CXCR4, CXCR5, and CCR7 on peripheral blood lymphocytes was significantly altered in patients with primary antibody deficiency compared with healthy controls.[35] Naive ($CD27^-$) and memory ($CD27^+$) B cells in study group both showed significantly lower percentages of cells positive for CXCR5 and CCR7.[35] There was no clear relationship between abnormal chemokine expression and any clinical complication.[35] Fevang et al.[36] extended previous reports on dysregulation of CCR7 expression in CVID and reported raised circulatory levels of its ligands CCL19 and CCL21, as well as an attenuated modulatory response to stimulation with these chemokines. In our study, Th1 (CCR5) and Th2 (CCR4) type chemokine receptors did not show any dominance in CVID cases (Table 5). However, as in the studies by Payne et al.[35] and Faveng et al.,[36] frequencies of CCR7 expressing $CD3^+$ T cells, $CD3^+CD4^+$ T helper cells, and $CD3^+CD8^+$ T cytotoxic cells were significantly lower in severe CVID patients

(Table 5). Moratto et al. also showed that the expressions of CXCR5 and CCR7 were reduced in a subset of CVID patients.[37] In addition, presence of autoimmune clinical findings was negatively correlated with CCR7$^+$ cells (Table 6). All of these findings support each other and the probable role of CCR7$^+$ cells in CVID.

The characterization of cell populations was not made at diagnosis in all patients, but it was made at follow-up, when certain complications were already established. It is not therefore so easy to establish a cause–effect correlation between Th1–Th2 polarization and complications.

In conclusion: (1) Our findings did not show reduced proportion of Tregs in CVID patients. Evaluation of other markers related to function of Tregs may provide additional data to clarify whether Tregs play a role in immune dysregulation in CVID disease. (2) Our data showed that severe CVID patients had abnormal chemokine receptor (CCR7) expression compared to healthy controls. As CCR7 is a key mediator balancing immunity and tolerance in the immune system, the abnormality of this mediator might contribute to the profound immune dysregulation seen in CVID. Whether this abnormality is primary or secondary to other factors contributing to the disease remains to be determined. (3) Finally, we compared cytokine production in cells from CVID patients and healthy children and we may speculate Th1 cells seem to be more involved in disease pathogenesis than Th2 cells.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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