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Study Protocol

Evaluation of B Cell and T Cell Phenotypes in CVID Patients and Its Correlation with the Clinical Phenotypes: Study Protocol

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Abstract

Background: Common variable immunodeficiency (CVID) is the most frequent symptomatic primary immunodeficiency, which manifests a wide range of clinical phenotypes from recurrent infections of the respiratory system to autoimmunity, enteropathy and lymphoproliferative disorders. Some abnormalities in T and B lymphocyte subpopulations may associate with the development of such clinical complications.

Aim of study: The main objective of this case-control study is to investigate the frequency and absolute count of different lymphocyte subsets in CVID patients as well as the cellular proliferation response. Correlation between lymphocyte abnormalities and different clinical phenotypes of the disease such as infection only (IO), autoimmunity (AI), chronic enteropathy (CE) and lymphoproliferative disorders (LP) are determined. We also aim to evaluate the prognosis of CVID for each clinical manifestation based on lymphocyte phenotype.

Methods: A population of genetically unsolved CVID patients after whole exome sequencing (WES) will be subdivided into 4 clinical phenotypes i.e. IO, AI, CE and LP and an equal number of age and sex-matched healthy controls (HC) will be examined for the frequency of distinct subgroups of $CD19^+$ B cells, $CD4^+$ T cells and $CD8^+$ T cells by flow cytometry. The proliferation response of their $CD4^+$ T cells is then evaluated by Carboxyfluorescein succinimidyl ester (CFSE) test, using stimulation of isolated peripheral blood mononuclear cells with anti-CD3 and anti-CD28 antibodies. Data analysis will be assessed by parametric or nonparametric tests based on normality of data distribution using IBM SPSS Statistics, V.24 and Stata software V.14.

Ethics and dissemination: Ethical approval of this study is received from the Ethics Committee of Tehran University of Medical Sciences (ID number: IR.TUMS.VCR.REC.1396.3380) and all participants will be asked to sign the informed consent statement. Due to the wide range of variables, objectives and questions, the findings of this study are intended to release as multiple publications in peer-reviewed journals and presented at national and international conferences.

Keywords: Primary Immunodeficiency; Common Variable Immunodeficiency; Immunologic Profile; Clinical Phenotypes; Proliferation Response

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Introduction

Common variable immunodeficiency (CVID) is one of the most common primary immunodeficiencies (PIDs) characterized by hypogammaglobulinemia, inability to produce specific antibodies in response to vaccination and recurrent infections with a relative prevalence of 1:10,000 to 1:50,000 (1). There are some mutations in the genes related to B cell receptor and activation which has been identified as the main cause of disease in 20-50% of the patients (2, 3). These mutations called the monogenic defects, can develop the same clinical symptoms as CVID, which are categorized as distinct PIDs with a CVID-like phenotype (4, 5). However, the remaining majority of clinically diagnosed CVID patients show no molecular defects and the real cause of disease in them remains a mystery.

Defects in B cell differentiation to plasma cells and memory B cells lead to a decreased antibody production and may result in increased susceptibility to recurrent bacterial infections, especially in the respiratory and gastrointestinal tracts (6, 7). However, some patients would show other clinical manifestations rather than infections, including autoimmunity, enteropathy, lymphoproliferative disorders, malignancy, and allergy (8, 9). These non-infectious complications are of major importance, since the immune dysregulation and inflammatory conditions combined, can give rise to more severe morbidity and mortality (10).

The earliest reports on lymphocyte defects in the CVID patients were first shown to be distinct abnormalities in B cell subsets, such as marked decrease in total B cells, naive B cells, Switched Memory B cells (SMB), Marginal Zone B cells (MZB) and plasmablasts along with an increase in $CD21^{low}$ and Transitional (Tr) B cells (11-16). Several different classifications of CVID patients were suggested based on the B subsets defects (13, 14, 17). Moreover, correlation of aforesaid alterations with different clinical phenotypes has been frequently reported; significant reduction in SMB and plasmablasts in patients with bronchiectasis, sinusitis and otitis media and also autoimmune disease (18-20), noteworthy rise in $CD21^{low}$ B cells in CVID patients with autoimmunity (21, 22), increase in Tr in correlation with lymphadenopathy (14, 23), a considerable decrease in SMB and MZB in CVID patients suffering from granulomatous disease or autoimmune cytopenia as well as splenomegaly (14-16) have been documented.

Considering the essential role of T cell help in the differentiation of B cells to antibody-producing plasma cells when exposed to T-dependent antigens, much pieces of evidence have provided data on T cell abnormalities in CVID patients (24-27). Such abnormalities, included not only follicular $CD4^+$ T cells but also, $CD8^+$ compartment and showed significant associations with the development of various clinical manifestations among the CVID patients. Several studies reported lymphopenia in $CD4^+$ T cells and decreased naive $CD4^+$ T cells in correlation with splenomegaly, autoimmunity and polyclonal lymphoproliferation (10, 28-30), decreased naive $CD8^+$ T cells in autoimmunity (30), reduced number and function of regulatory T cells (Treg) in autoimmunity and splenomegaly accompanied by a rise in $CD21^{low}$ B cells (30-32), reduction in T helper 17 (Th17) and rise in Th1 in enteropathy (33, 34), increase inactivated and cytotoxic $CD8^+$ T cells with autoimmunity (35) and an excessive number of $CD8^+$ Effector Memory (EM) and Terminally Differentiated Effector Memory (T_{EMRA}) T cells in the CVID patients with lymphoproliferative disorders (36, 37). Several investigations also reported defects in the proliferation response of T cells to antigens and mitogens (38-41).

This study aims to evaluate the complete B cell and T cell subsets in genetically unsolved CVID patients and age- and sex-matched Healthy Controls (HC) in addition to exploring the observed abnormalities in correlation with different clinical phenotypes of CVID.

Objectives and research questions

The current study is designed to address several questions regarding the association between CVID and lymphocyte subpopulations. The primary objective of this study is to determine the correlation between the percentage and count of B cell and T cell subsets and the prognosis of CVID as well as different clinical phenotypes observed among the CVID patients. The secondary objectives include evaluation of T cell function in association with CVID and

its different clinical phenotypes together with the comparison of various CVID classification systems for our patients.

This study will intend to address the following questions:

1. How does the percentage and count of the $CD19^+$ B lymphocyte subpopulations in CVID patients, differ between Infections-Only (IO), Autoimmunity (AI), Chronic Enteropathy (CE) and Lymphoproliferation (LP) phenotypes?

2. How does the percentage and count of the $CD4^+$T lymphocyte subpopulations in CVID patients, differ between IO, AI, CE and LP phenotypes?

3. How does the percentage and count of the $CD8^+$T lymphocyte subpopulations in CVID patients differ between IO, AI, CE and LP phenotypes?

4. How does the proliferation response of $CD4^+$T lymphocytes of the CVID patients, differ between IO, AI, CE and LP phenotypes?

5. What are the overlapping manifestations and associations between lymphocyte subsets and proliferation alterations in each specific clinical phenotype?

6. What are the prognoses of the CVID and each complication based on the B and T cell subpopulations?

7. How does the percentage and count of the B and T lymphocyte subpopulations change in the genetically unsolved CVID patients in comparison to previously reported monogenic CVID-like patients?

8. How many of patients, diagnosed initially with CVID, might have any evidence of profound T cell deficiency and be considered as Unclassified Combined Immunodeficiency (CID) sufferer?

Methods and Analysis

Study design

In order to address these questions, we will design a case-control study in Iran based on the CVID patients who have no known mutation in their Whole-Exome Sequencing (WES) analysis. The data of the cases will be collected from the Iranian PID registry (42) and also will be extracted from the questionnaire which will be designed specifically for this research.

Participants

Inclusion and exclusion criteria

Patients who will be included in this study are adult CVID patients with the age of 16 years and more in both sexes who are referred to Children's Medical Centre affiliated to Tehran University of Medical Sciences. The clinical diagnosis of CVID will be established based on the criteria proposed by the European Society for Immunodeficiencies (43, 44). They are also confirmed to have no definitive genetic mutations according to the WES analysis. Patients with a mutation within all known monogenic PID genes will be excluded from this study (45-47). Based on clinical examination, the paraclinical tests and imaging, besides the previously recorded information on the registry, patients will be categorized into 4 distinct clinical phenotype groups; IO, AI, CE and LP (48). Equal numbers of age- and sex-matched individuals with no history of respiratory infections or immunologic diseases such as allergy, asthma or autoimmunity and malignancy will comprise the HC (healthy control) group.

Data collection

To collect data for this case-control study, we will need to conduct two types of laboratory tests, including extracellular and intracellular flow cytometry techniques for the detection of lymphocyte subpopulations and also CFSE (Carboxyfluorescein Succinimidyl Ester) test for the evaluation of T cells' proliferation response.

Table 1. Surface antibodies for the detection of B cell subsets and related fluorescent dyes conjugated

Tube	FITC	PE	PerCP-eFluor710	APC
B1	Anti-CD27	Anti-IgD	Anti-IgM	Anti-CD19
B2	Anti-CD38	Anti-CD21	Anti-IgM	Anti-CD19

FITC, fluorescein isothiocyanate, PE, phycoerythrin, APC, allophycocyanin, PerCP, peridinin chlorophyll protein

Table 2. Surface antibodies for the detection of naive and memory T Cell subsets and related fluorescent dyes conjugated

Tube	FITC	PE	PerCP-Cyanine5.5
T4	Anti-CD45RA	Anti-CD197 (CCR7)	Anti-CD4
T8	Anti-CD45RA	Anti-CD197 (CCR7)	Anti-CD8a

Laboratory Methods

Flow cytometry analysis

Based on extracellular flow cytometry, several steps for the detection of B and T cell subpopulations will be performed, which are as follows:

1. Peripheral blood mononuclear cells (PBMC) are isolated by Ficoll-Hypaque (Lymphosep, Biosera, France) density gradient centrifugation.

2. For the detection of B cell subsets, PBMCs are washed with the flow buffer (Phosphate Buffered Saline [PBS] (Sigma, MO, USA) containing 2% Fetal Bovine Serum [FBS] (Biosera, France)) and after dividing into two tubes named B1 and B2, the cell-surface antibodies are added to each tube according to **Table 1**. They then incubated for 30 minutes at 4ºC in the dark.

3. By using the aforementioned antibodies, in the tube B1 we can detect naive, MZB, SMB and IgM only MB and in the tube B2 we will recognize Tr, $CD21^{low}$ and plasmablasts, accordingly:

Naive B cell: $CD19^{+}$, $CD27^{-}$, IgM^{+} , IgD^{+}
Marginal zone B cell: $CD19^{+}$, $CD27^{+}$, IgM^{++}, IgD^{+}
IgM only memory B cell: $CD19^{+}$, $CD27^{+}$, IgM^{++}, IgD^{-}
Switched memory B cell: $CD19^{+}$, $CD27^{+}$, IgM^{-}, IgD^{-}
Transitional B cell: $CD19^{+}$, $CD21^{+}$, $CD38^{++}$, IgM^{+}
$CD21^{low}$ B cell: $CD19^{+}$, $CD21^{-/low}$, $CD38^{-/low}$, IgM^{+++}
Plasmablast: $CD19^{+}$, $CD21^{-/low}$, $CD38^{++/++}$, IgM^{-}

4. For the detection of naive and memory T cell subsets, PBMCs are washed with the flow buffer and after dividing into two tubes named T8 and T4, the cell-surface antibodies are added to each tube according to **Table 2**. They then incubated for 30 minutes at 4ºC in the dark.

5. By the usage of the aforesaid antibodies, in both the T4 and T8 tubes we can detect naive, Central Memory (CM), Terminally Differentiated Effector Memory (T_{EMRA}) and Effector Memory (EM) $CD4^{+}$ and $CD8^{+}$ T cells, respectively:

Naive: $CD45RA^{+}$, CD197 $(CCR7)^{+}$
CM: $CD45RA^{-}$, CD197 $(CCR7)^{+}$
T_{EMRA}: $CD45RA^{+}$, CD197 $(CCR7)^{-}$
EM: $CD45RA^{-}$, CD197 $(CCR7)^{-}$

6. For the detection of Activated $CD8^{+}$ T cells, PBMCs are washed with the flow buffer and then anti-CD8α (PerCp-Cy5.5), anti-HLA-DR (PE) and anti-CD38 (FITC) antibodies are added. They are then incubated for 30 minutes at 4ºC in the dark.

7. After 30 minutes of incubation of the tubes in the dark at 4ºC, cells are washed with the flow buffer and counts are determined using a Flow Cytometer. Gating and analyzing of results are done by FlowJo software (Tree Star, Ashland, OR, USA).

On the other hand, there are several steps for the detection of T cell subpopulations based on intracellular flow cytometry, which are as follows:

1. For the detection of helper T cells (Th), PBMCs are stimulated with Phorbol Myristate Acetate (PMA) (Sigma, MO, USA) and Ionomycin Calcium Salt (Sigma, MO, USA) for 5 hours in RPMI (Roswell Park Memorial Institute) culture medium (Biosera, France) containing 10% FBS at 37ºC in a 5% CO_2 humidified incubator in the presence of Brefeldin A (eBioscience, CA, USA).

2. The stimulated PBMCs are harvested from plates and washed with PBS and after that with the flow buffer. Then the anti-CD4 (PerCp-Cy5.5) antibody is added and incubated for 30 minutes at 4ºC in the dark.

3. Cells are washed, fixed and permeabilized with fixation/permeabilization buffer (eBioscience, CA, USA), suspended in the permeabilization buffer (eBioscience, CA, USA). Afterwards, antibodies to intracellular cytokines; the anti-IFN-γ (FITC), anti-IL-17 (PE) and anti-IL-4 (APC) antibodies are added and incubate for 30 minutes at room temperature in the dark.

4. For the detection of regulatory T cells (Treg), unstimulated PBMCs are washed with the flow buffer and the anti-CD4 (PerCp-Cy5.5), anti-CD25 (APC) and anti-CD127 (FITC) antibodies

are added. They are then incubated for 30 minutes at 4ºC in the dark.

5. Cells are washed, fixed and permeabilized with fixation/permeabilization buffer, suspended in the permeabilization buffer. Afterwards, an intracellular anti-FOXP3 (PE) antibody is added and incubated for 30 minutes at room temperature in the dark.

6. For the detection of Cytotoxic T cells (CTL), unstimulated PBMCs are washed with flow buffer and an anti-CD8α (PerCp-Cy5.5) antibody is added. They are then incubated for 30 minutes at 4ºC in the dark.

7. Cells are washed, fixed and permeabilized with fixation/permeabilization buffer and suspended in the permeabilization buffer. Afterwards, an intracellular anti-granzyme B (PE) antibody is added and incubated for 30 minutes at room temperature in the dark.

8. After incubation in the dark and room temperature, cells are washed with permeabilization buffer and the counts are determined using a Flow Cytometer. Gating and analyzing of results will be done by FlowJo software.

The isotype-matched controls will be applied in both the extracellular and intracellular methods to determine if there is any non-specific background staining for the primary antibodies. All the antibodies and isotype controls for flow cytometry tests will be purchased from eBioscience, CA, USA.

CFSE Test

Coating the plates

1. For each sample, one well from a sterile 24-well, tissue culture plate is precoated with anti-CD3 antibody (eBioscience, CA, USA) at 1 µg/ml diluted in PBS as stimulated and one well is just filled with equal volume of PBS as unstimulated.

2. After 2 hours of incubation in a humidified atmosphere at 37ºC, 5% CO_2, each well is washed with PBS two times.

CFSE staining/labelling

1. PBMCs are resuspended in PBS containing 5% FBS and then an equal volume of 10µM CFSE (Biolegend, CA, USA), which is diluted in PBS, is added to reach a final concentration of 5µM for CFSE. The cells are then incubated for 5 minutes in a humidified atmosphere at 37ºC in the dark.

2. After quenching the staining by adding 10 ml of RPMI containing 10% FBS, the cells are washed with this medium three times.

3. The PBMCs stained with CFSE is added to both wells at a final concentration of 2×10^6 /ml in RPMI containing 10% FBS.

4. After adding the anti-CD28 antibody (eBioscience, CA, USA) at 2 µg/ml to the stimulated well, plates are incubated for 4 days in a humidified atmosphere at 37ºC, 5% CO_2.

Cell harvesting

1. On day 4, the cells are harvested by vigorous pipetting into two flow cytometry tubes named stimulated and unstimulated and are washed with flow buffer.

2. The anti-CD4 (PerCp-Cy5.5) antibody is added to each tube and incubates for 30 minutes at 4ºC in the dark.

3. After incubation in the dark and 4ºC, cells are washed with flow buffer and the counts are determined using a flow cytometer. Gating, comparison of stimulated and unstimulated cells and analyzing of results are done by FlowJo software using proliferation modeling platform.

Statistical analysis

Following descriptive analyses, values will be expressed as mean ± standard error of the mean (SEM) of absolute count (/µl) and percentage (%). The One-Sample Kolmogorov-Smirnov or Shapiro-Wilk Test is used to check normality of the results. According to normality results, Mann–Whitney U test or Independent-Samples T-test, Paired-Samples T-Test or Wilcoxon Signed Ranks Test, Pearson Correlation Test or Spearman Ranks will be selected for comparison between cases and controls, within groups and various correlations, respectively. These analyses will be operated by IBM SPSS Statistics for Windows, version 24 (IBM Corp, Armonk, NY, USA). Moreover, to evaluate the Odds Ratio (OR), the Conditional Logistic Regression analysis will be applied using Stata (Stata-Corp. 2015. Stata Statistics Software: Release 14. College Station, TX, USA: StataCorp LP).

Dissemination plan

We plan to disseminate the research findings, not only as printed articles in the academic

and clinical peer-reviewed journals, but also as conference presentations. Considering the enormous, expected variables' generation and designed objectives for this study, we need to release the results in multiple publications.

Discussion

In some CVID patients, the development of clinical manifestations, such as infections, enteropathy, autoimmunity and lymphoproliferative disorders, will lead to more severe conditions of disease, morbidity and mortality. However, alterations in the B and T cell compartments could be linked to the pathogenesis or the underlying causes of these clinical manifestations. Although many investigations have shown the link between various abnormalities in the lymphocyte subsets and distinct clinical phenotypes of heterogeneous CVID patients without any genetic evaluation, we decide to conduct a comprehensive study to evaluate both the humoral and cellular immunity simultaneously in correlation with the adverse clinical presentations only on patients without any monogenic defects.

Our research will not only clarify the frequency of the B and T cell subpopulations as well as T cell proliferation response in the CVID patients with no definitive genetic mutations, but also demonstrate the potential role of the lymphocyte abnormalities in the development of their clinical features. Moreover, the risk of developing CVID distinct clinical phenotypes, can be determined based on the lymphocyte phenotype, in addition to establishing the associations between the lymphocyte alterations. This study will also help to determine a prognosis of the clinical symptoms, reduce diagnostic delays and establish a differential diagnosis between CID and CVID.

Strengths and limitations of this study

This study is the first to conduct a comprehensive research on the CVID patients with no definitive genetic mutations according to the WES analysis, in order to compare the advance immunologic profile of the distinct clinical phenotypes. Additionally, it may discover the risks and potential correlations required for the disease prognosis estimation.

This research will use a case-control design, by which we will be efficiently capable of tackling the research questions. To lessen the effects of the main genetic factors responsible for the etiology of CVID, all cases involved in this study are exclusively patients with no known mutations in WES in combination with the matching of sex and age of controls with the cases.

Accordingly, it can lead to a decreased sample size in some clinical phenotype groups and still the environmental and epigenetic modifying factors can be different.

One of the limitations of this study is that, most of our cases are likely to regularly receive immunoglobulin replacement as their routine treatment or other medications, which may cause our study to end up converting to a prevalent case-control rather than an incident case-control with a more study power.

Ethical Approval

Ethical approval of this study is received from the Ethics Committee of Tehran University of Medical Sciences (ID number: IR.TUMS.VCR.REC.1396.3380) and all participants will sign the informed consent statement before the recruitment to the study.

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Conflict of interest

The authors declare no conflicts of interest.

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