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Decreased number of regulatory T lymphocytes is related to inflammation and number of CD8$^+$ T cells expressing programmed cell death protein-1 in common variable immunodeficiency

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Abstract: Common variable immunodeficiency (CVID) is a primary immunodeficiency disorder related to recurrent infections, as well as a range of non-infectious manifestations including autoimmune and inflammatory disorders. We hypothesized that patients with CVID and different clinical phenotypes would demonstrate alterations in lymphocyte T subsets, including T lymphocytes expressing programmed cell death protein 1 (PD-1), and regulatory T lymphocytes. We performed flow cytometry in two CVID groups: group 1 with infections only, and group 2 with infections and concomitant noninfectious manifestations. Patients were 18–59 years old (mean 35.8 years of age). Increased proportions of CD8$^+$PD-1$^+$ T cells and reduced regulatory T cells were associated with lymphadenopathy. Amount of regulatory T cells correlated with CD8$^+$PD-1$^+$ T lymphocytes ($r = 0.54$; $p = 0.013$), and with CRP ($r = -0.64$; $p = 0.004$). Forty percent of patients expressed manifestations in addition to infections (group 2), and they had reduction in number of regulatory T cells [8 (3–12) vs. 24 (11–26)/μl; $p = 0.034$), naive CD4$^+$ T lymphocytes [36 (27–106) vs. 149 (81–283)/μl; $p = 0.034$], and elevated C-reactive protein (CRP) [5.33 (3.15–8.82) vs. 1 (1–2.16) mg/l; $p = 0.003$] in comparison to group 1. In conclusion, the amount of CD8$^+$ T cells expressing PD-1 is associated with lymphadenopathy and number of regulatory T cells in patients with CVID. Patients with CVID and non-infectious complications have increased level of inflammation and alterations in regulatory T cells.

Keywords: common variable immunodeficiency, T cells, regulatory T cells, PD-1/PD-Ls, chronic inflammation.

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Introduction

Common variable immunodeficiency disorder (CVID) is a primary immunodeficiency, a rare disease highly diverse in clinical manifestations, and characterized by hypogammaglobulinemia [1]. Although several monogenic forms of CVID have been described, the mode of inheritance is mostly polygenic [1, 2]. Clinical manifestations of CVID include primarily recurrent sinopulmonary infections, but patients may also present increased predisposition to development of cancer, autoimmunity or inflammatory disorders [1]. Autoimmune disorders are diagnosed in about 20–25% of CVID patients [3]. In a recent retrospective study of patients with PIDs by Fischer *et al.* [4], survival time was significantly shorter in subjects with autoimmune and/or inflammatory manifestations than in patients without these manifestations. Moreover, in that population the risk of autoimmune cytopenia was significantly higher in comparison with general population. In addition, in Fischer's report, inflammatory bowel disease and arthritis were among the most frequent autoimmune/inflammatory diseases and subjects with CVID and T-cell deficiencies demonstrated the highest risk [4]. Various mechanisms of immune dysregulation, abnormalities in both B and T lymphocytes leading to non-infectious complications in CVID have been proposed. For instance, mutations in the gene encoding nuclear factor κB subunit 1 (NF-κB1), reduced NF-κB1 protein levels, and expanded $CD21^{low}$ B-cell subset have been demonstrated in patients with CVID and inflammatory bowel disease, arthritis or Behçet disease, compared with healthy controls subjects [5]. In addition, aberrations in T lymphocytes, including decreased $CD4^+$ lymphocytes, regulatory T cells, and Th17 cells have also been suggested, and were associated with lymphoproliferative complications in some studies [6–9]. To our best knowledge, the role of the programmed cell death protein 1 (PD-1) has not been extensively evaluated in CVID. PD-1 exerts a suppressive effect on self-reactive T cells, and may also be engaged in the suppression of effector T lymphocytes, and therefore may have negative effects on antiviral and anticancer response [10–12]. The purpose of our research, therefore, was to assess lymphocyte T subsets, including $CD8^+$PD-1 and regulatory T lymphocytes in CVID patients in relation to clinical manifestations.

Materials and Methods

The diagnosis of CVID was based on the European Society for Immunodeficiencies (ESID) criteria [13]. We enrolled 20 adult patients with CVID receiving regular immunoglobulin replacement therapy every 3 to 4 weeks. Patients were included in the study after informed written consent was obtained from each individual, the study was conducted according to the approval by the local institutional ethics committee and in accordance with the 1964 Helsinki declaration and its later amendments. None of the patients received immunosuppressive therapy other than a low dose of glucocorticoids

(less than or equal to 7.5 mg prednisone per day — 3 cases) at the time of the study and in 6 months prior the study. Data such as number of infections, concomitant diseases, IG dose, and age at diagnosis were collected retrospectively. Patients were divided into two clinical phenotypes: group 1 including patients diagnosed with CVID, and with infections as a principal clinical manifestation, and group 2 including patients with CVID, infections and associated inflammatory complications including gastrointestinal diseases (inflammatory bowel disease, celiac disease, primary biliary cholangitis), psoriasis, psoriatic arthritis, and diabetes type 1. Additionally, patients in group 2 had clinical manifestations including splenomegaly, lymphadenopathy, and cytopenias.

Laboratory studies

All laboratory tests were performed on blood samples taken directly before consecutive IG administration, and basic tests included: complete blood count with differential, glucose, creatinine, alanine aminotransferase (ALT), asparaginian aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGTP), bilirubin, thyrotropin releasing hormone (TSH), total protein, albumin, iron, ferritin, total iron binding capacity (TIBC), IgG, IgA, IgM and C-reactive protein (CRP). An abdominal ultrasound exam was also performed to evaluate spleen size.

Peripheral blood samples were incubated with monoclonal fluorescent-antibodies in order to evaluate surface antigen expression among lymphocyte populations (including anti-CD3-FITC, anti-CD4-Alexa Fluor 700, anti-CD8-APC-H7, anti-CD25-PE, anti-CD27-BC510, anti-CD28-PerCP-Cy5.5, anti-CD127-BV605, anti-CD279-Alexa Fluor 647, anti-CD45RA-PE-Cy7, anti-HLA-DR-PerCP, anti-CCR7-BV421 obtained from BD Biosciences, San Jose, CA, USA) using flow cytometry. The samples were then treated with FACS Lysing Solution (BD), washed twice with PBS, handled in the FACSCanto 10 color flow cytometer (Immunocytometry Systems, BD), and evaluated with FACSDiva Software (BD). The absolute numbers of lymphocyte subpopulations were determined applying dual-platform method. The absolute lymphocyte count was derived from a hematology cell analyzer ABX Micros ES 60 Horiba (Horiba Medical, Irvine, CA, USA). Lymphocytes were gated according to forward scatter (FSC) and side scatter (SSC) signals from PBMC and T cells were gated according to CD3 expression. Percentages and numbers of CD4 and CD8 positive subpopulations were assessed. T cell subpopulations were also analysed for the presence of naïve (N) T lymphocytes ($CD45RA^{+}CCR7^{+}CD\ 28^{+}CD27^{+}CD127^{+}CD279^{-}$), effector memory (EM) T cells ($CD45RA^{-}CCR7^{-}CD28^{+}CD27^{+/-}CD127^{+}CD279^{+}$), central memory (CM) T cells ($CD45RA^{-}CCR7^{+}CD27^{++}CD28^{++}CD127^{++}CD279^{-}$), terminally differentiated effector memory (TEMRA) T cells ($CD45RA^{+}CCR7^{-}CD\ 27^{-}CD28^{-}CD127^{+}CD279^{+/-}$), activated T cells (CD3/HLADR), and regulatory T cells ($CD4^{+}CD25^{++}CD127^{-}$). PD-1 expression (CD279) was assessed on $CD4^{+}$ and $CD8^{+}$ T lymphocytes.

Statistical analyses

Results are presented as means (standard deviation, SD), medians (interquartile range, IQR) for continuous variables, and percentages for categorical variables. The conformity with a normal distribution was tested by the Kolmogorov–Smirnov test and uniformity of variances by Leven's test. Intergroup differences were calculated applying the unpaired 2-sided Student's t-test or Mann–Whitney U test, and Fisher's exact test for continuous variables and proportions, respectively. Bivariate correlations between continuous variables were estimated by Spearman's correlation coefficient (r). All presented results were considered significant at p value below 0.05. Data were analyzed with Statsoft Statistica® software (statsoft Inc., STATISTICA data analysis software system, version 13).

Results

The amount of patients included in the study was 20, with 11 (55%) males; aged 18–59 years. Mean age of diagnosis of CVID was 27.5 ± 15.5 years, 7 patients were diagnosed before 15 years of age. Immunoglobulins were administered intravenously in 17 patients and subcutaneously in 3 patients, with an average dose of 0.45 ± 0.13 g per kilogram of body weight. Among 20 patients, 12 (60%) presented with infections only (group 1), and 8 (40%) presented both with infections and other CVID associated noninfectious conditions (group 2), i.e. there were 5 cases of gastrointestinal inflammatory diseases, 1 case of primary biliary cholangitis, 1 case of diabetes type 1, and 1 case of psoriatic arthritis. Additionally, in group 2 any cytopenia was found in 8 subjects, lymphadenopathy and splenomegaly in 5 and 11 patients, respectively. Basic clinical findings are presented in Table 1 and Table 2. Patients from group 2 had significantly lower IgG serum levels despite comparable monthly IG dose, and elevated CRP levels as well as higher ALT and ALP levels compared with group 1.

Table 1. Clinical characteristics of CVID patients according to disease manifestations.

	Group 1 infections-only n = 12	Group 2 non-infectious complications n = 8	p
Age (years)	33.5 ± 13.1	39.1 ± 12.3	0.35
Age at CVID diagnosis (years)	25.6 ± 15.7	30.4 ± 15.9	0.51
Female sex (%)	50	37.5	0.67
Ig dose (g/kg)	0.41 ± 0.11	0.53 ± 0.14	**0.05**
IgG at diagnosis (g/l)	3.85 (1.44–5.24)	1.03 (0.37–2.8)	0.14
IgG (g/l)	8.00 ± 1.30	5.80 ± 1.50	**0.003**

IgA (g/l)	0.24 (0.24–0.25)	0.25 (0.24–0.22)	0.65
IgM (g/l)	0.28 (0.17–0.59)	0.17 (0.17–0.27)	0.34

Data are shown as the means ± SD or the medians and the (25th and 75th) percentiles.

Table 2. Basic laboratory tests findings according to CVID manifestations.

	Group 1 infections-only n = 12	Group 2 non-infectious manifestations n = 8	p
CRP (mg/l)	1 (1–2.16)	5.33 (3.15–8.82)	0.003
Leukocytes (10^9/L)	5.87 ± 1.88	8.5 ± 4.53	0.23
Erythrocytes (10^{12}/L)	5.02 ± 0.44	4.8 ± 0.6	0.27
Haemoglobin (g/L)	138 ± 17	136 ± 18	0.71
Hematocrit (L/L)	0.41 ± 0.04	0.40 ± 0.04	0.51
Platelets (10^9/L)	210.5 ± 69.8	216.4 ± 153.4	0.48
Neutrophils (10^9/L)	3.07 ± 1.2	5.14 ± 2.91	0.09
Lymphocytes (10^9/L)	1.95 ± 0.8	2.45 ± 2.22	1
Monocytes (10^9/L)	0.63 ± 0.27	0.76 ± 0.5	0.77
AST (U/l)	25 (17–32)	29.5 (24.5–38.5)	0.25
ALT (U/l)	16 (13–29)	35 (32.5–51)	0.015
LDH (U/l)	358 ± 112.3	422.9 ± 140.2	0.17
GGTP (U/l)	11.5 (10–28)	21 (16–31)	0.30
ALP (U/l)	80.3	117.88	0.037
Glucose (mmol/l)	4.47 ± 0.63	5.2 ± 1.46	0.40
TSH (uIU/ml)	2.36 ± 1.09	2.25 ± 1.16	1
Creatinine (μmol/l)	64.7 ± 11.6	74.5 ± 34.5	0.79
CK (U/l)	112 (90–159)	64 (48–173)	0.24

Data are shown as the means ± SD or the medians and the (25th and 75th) percentiles.

The results of the analysis of T lymphocyte subsets are presented in Table 3. White blood cell count and lymphocyte count were comparable in both groups. Within T lymphocytes, the number of naive CD4 T cells (median 447 cells/μl) and regulatory T cells (median 8 cells/μl) were substantially lower in group 2 in comparison to group 1 (median 679 cells/μl; p = 0.034 and 24 cells/μl; p = 0.034), respectively. Remaining T lymphocyte subsets, including numbers of T cells expressing PD-1 were comparable in both groups (Fig. 1).

Table 3. T cell subsets according to CVID manifestations.

	Group 1 infections-only n = 12	Group 2 non-infectious complications n = 8	p
Lymphocytes (/μl)	1850 (1300–2300)	1660 (1000–3250)	0.88
CD4 (/μl)	679 (409–906)	447 (314–881)	0.33
CD4 naive (/μl)	149 (81–283)	36 (27–106)	0.03
CD4 central memory (/μl)	110 (83–138)	80 (49–144)	0.46
CD4 effector memory (/μl)	63 ± 45	94 ± 72	0.30
CD4 TEMRA (/μl)	0.4 (0.1–4)	2 (0.2–9)	0.79
Regulatory T cells (/μl)	24 (11–26)	8 (3–12)	0.03
CD4 PD-1 (/μl)	240 ± 147	254 ± 117	0.82
CD8 (/μl)	613 (371–869)	952 (264–1553)	0.56
CD8 naive (/μl)	124 (56–160)	43 (11–119)	0.15
CD8 central memory (/μl)	30 (18–50)	26.8 (10.4–48.1)	0.96
CD8 effector memory (/μl)	17.8 (0.5–51.4)	20 (7–34)	0.91
CD8 TEMRA (/μl)	12 (6–20)	20 (7–26)	0.56
CD8 PD-1(/μl)	205 (133–341)	320 (133–341)	0.56

Data are shown as the means ± SD or the medians and the (25th and 75th) percentiles.

Splenomegaly was related to increased percentage of $CD4^+$ lymphocytes (49.5 ± 10.2 vs. 34.5 ± 11.8%; p = 0.007) and reduced percentage of $CD8^+$ lymphocytes (median 36.5 vs. 50.1%; p = 0.008). Lymphadenopathy, was related to elevated CRP level (median 7.02 vs. 1.62 mg/l; p = 0.02), lower percentage of naïve $CD4^+$ T cells (median 2.2 vs. 11.6%; p = 0.003), central memory $CD4^+$ lymphocytes (median 4.6 vs. 9.2%; p = 0.003), higher proportion of $CD8^+$ cells (66.4 ± 14.7 vs. 40.9 ± 9.0%; p <0.001), elevated percentage of $CD8^+$ lymphocytes expressing PD-1 (median 24 vs.14.1%; p = 0.001), and diminished proportion of regulatory T cells (0.5 vs. 1.2%; p = 0.009) (Fig. 2). Malignancy was confirmed in 1 patient (non-melanoma skin cancer). In the entire group of CVID patients, regulatory T cell number correlated negatively with CRP (r = –0,64; p = 0.004), and was positively associated with counts of $CD8^+PD\text{-}1^+$ T lymphocytes (r = 0.54; p = 0.013), and $CD4^+$ naive T cells (r = 0.73; p <0.001) (Fig. 3).

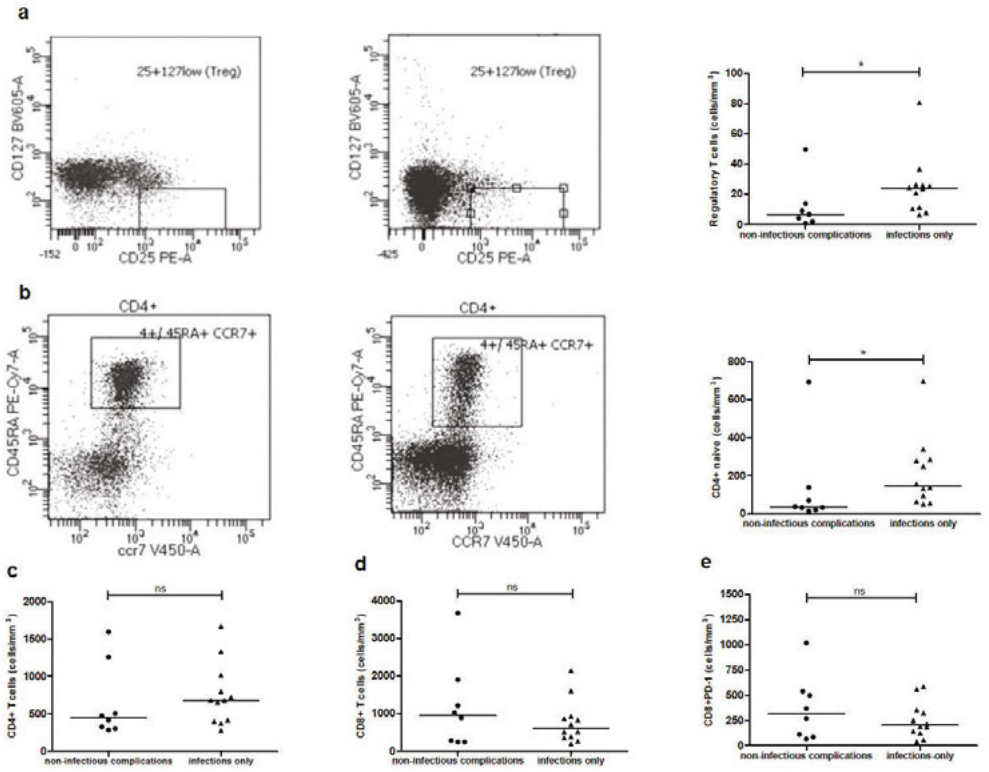

Fig. 1. Comparison of T cells and subsets in two groups of CVID patients with non-infectious complications and infections-only. Representative plots with cells obtained from a CVID patient with disease associated complications (left panel a and b), and a CVID patient with infections only (right panel a and b) showing decreased number of regulatory T (Treg) cells (a), and naive $CD4^+$ T cells (b), comparable numbers of $CD4^+$, $CD8^+$ and $CD8^+$ PD-1^+ T cells (c–e). Horizontal lines represent the medians. *p <0.05.

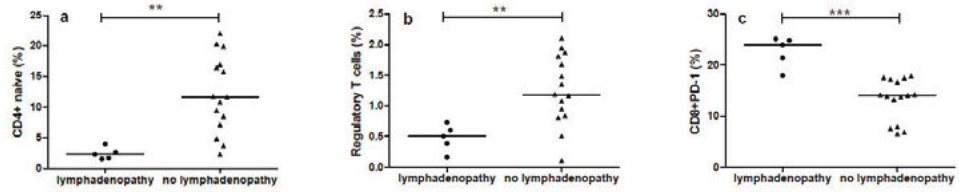

Fig. 2. Lymphadenopathy in CVID was associated with decreased percentage of regulatory T cells, naïve $CD4^+$ T cells and increased proportion of $CD8^+PD\text{-}1^+$ expressing T cells. Horizontal lines represent the medians. **p <0.01, ***p <0.001.

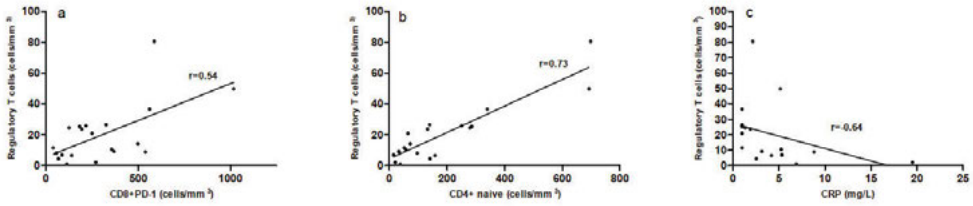

Fig. 3. Reduced number of regulatory T cells in CVID correlated with decreased number of $CD8^+PD\text{-}1^+$ T cells (a); $p < 0.05$, naive $CD4^+$ T cells (b); $p < 0.001$, and elevated C-reactive protein.

Discussion

We have demonstrated the positive correlation among the number of $CD8^+$ T lymphocytes with PD-1 expression and regulatory T lymphocytes. We did not observe any substantial differences in PD-1 expression on T cell subsets between the infection-only group and the group with noninfectious complications, nonetheless we demonstrated that patients with lymphadenopathy had nearly 1.5 times increased proportion of $CD8^+$ T cells expressing PD-1 in comparison with patients without lymphadenopathy. Our research is one of a very small number of studies evaluating PD-1 expression on T cells in CVID. We believe that this is the first work, that shows presence of relation between regulatory T lymphocytes and $CD8^+$ lymphocytes expressing PD-1 in CVID. We have also demonstrated decreased counts of regulatory T lymphocytes and naive $CD4^+$ T lymphocytes, as well as increased C-reactive protein serum level in patients with CVID and concomitant noninfectious complications in comparison to the infection-only group. In addition, we showed that in the pooled groups low regulatory T lymphocytes correlated with increased C-reactive protein level.

The PD-1/PD-L pathway regulates peripheral tolerance and it is involved in tumor immunity and chronic infections [11, 12]. Our results are consistent with findings of Kuntz *et al.*, who found that $CD8^+$ T lymphocytes of patients with CVID demonstrated more antigen experienced and activated differentiation state, especially in a group with lymphadenopathy and granulomatous disease [14]. It has been confirmed that in chronic infections PD-1 could be used as a marker of exhausted $CD8^+$ T cells with impaired function [15], but $CD8^+PD\text{-}1^+$ T cells are also found in healthy humans where PD-1 is a marker of the majority of effector memory T cells [16]. The mechanism of T cells exhaustion in CVID is not determined, but it may be related to chronic activation of CD8+ T lymphocytes and prolonged expression of PD 1 caused by recurrent infections [17]. Marashi *et al.* found increased proportion of CMV $CD8^+$ T lymphocytes in CVID patients, and high levels of interferon-γ and tumor necrosis factor-α, compared with healthy controls [18]. Autoimmunity may theoretically contribute to an excessive T cell response in CVID as well, although the role of autoimmunity is unproven [17]. The role of PD-1 in T lymphocyte impairment

has been studied in human with rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus, myasthenia gravis or chronic hepatitis C infection [19–25]. In chronic inflammatory diseases, chronic stimulation of $CD8^+$ T cell could initiate exhaustion similar to that observed in chronic infections [25]. Nevertheless, a study by Petrelli *et al.* demonstrated that $CD8^+$ $PD\text{-}1^+$ lymphocytes in synovia of patients with juvenile idiopathic arthritis were effector lymphocytes and were not exhausted cells [26]. Therefore, according to these authors, $PD\text{-}1^+$ $CD8^+$ T cells could be antigen experienced lymphocytes specific to certain sites of chronic inflammation. Data on PD-1 in CVID is scarce. De Lollo *et al.* found enlarged expression of PD-1 on effector memory $CD4^+$ T lymphocytes compared to healthy individuals, but not on $CD8^+$ lymphocytes [27]. Another study demonstrated, that in CVID the functional impairment of $CD4^+$ T cells was caused by PD-1 associated cell exhaustion, and in vitro CD4 proliferation was brought back by blocking PD-L1/2 [28]. Noteworthy finding of our study is the association of $CD8^+$ lymphocytes expressing PD-1 with regulatory T cells in CVID. As regulatory T lymphocytes produce inhibitory cytokines such as interleukin-10 or transforming growth factor β, they may contribute to $CD8^+$ T lymphocyte exhaustion [29]. Furthermore, reduction of regulatory T cells and blockade of PD-1 has common effect on reversal of exhaustion of $CD8^+$ T cells [30]. Therefore, decreased expression of PD-1 in CVID, resulting in increased T cell proliferation and cytokine production may well impair immune responses and hasten autoimmunity, which is associated with decreased regulatory T cells.

The finding of decreased regulatory T cells in CVID is in line with the previously published data [31–33]. Observed changes in regulatory T cells in CVID may represent abnormal immune effects responsible for the phenomenon of autoimmunity [34]. Most of the studies evaluating the number and mechanisms of modulation of regulatory T cells function during inflammation refer to chronic inflammatory diseases, which also may occur in the course of CVID. The exception was a study by Fevang *et al.* performed in CVID patients, who showed that serum level of neopterin was correlated negatively with the amount of regulatory T cells [32]. A study by van Amelsfort *et al.* reported that the number of peripheral $CD4^+CD25^{++}$ regulatory T lymphocytes in patients with rheumatoid arthritis was decreased as compared with the level found in their synovial fluid [35]. That phenomenon may be associated with the accumulation of regulatory T cells in tissues, and however not formally proven, it might be also relevant to the results of our study. The function of regulatory T cells may be varied in chronic inflammation, where impaired regulatory lymphocytes might contribute to activation of immune response and predispose to autoimmunity, as has been shown by Hguyen *et al.* [36].

We also found, that subjects with CVID and noninfectious complications presented additional T cell abnormalities, including decrease in naive $CD4^+$ T lymphocytes. The decline in naive $CD4^+$ T lymphocytes in CVID has been already described,

and it was shown to be associated with the severity of disease [37, 38], and with gastrointestinal symptoms or polyclonal lymphoproliferation [38]. Giovannetti *et al.* have previously classified CVID patients according to number of naive $CD4^+$ T lymphocytes, and the group with decreased number was characterized by T lymphocyte activation, proliferation, apoptosis, and splenomegaly [37]. In our study lymphadenopathy and low regulatory T cell number, but not splenomegaly, were related to lower level of naive $CD4^+$ lymphocytes.

A number of limitations of the study should be acknowledged. Firstly, the results are limited by the small size and heterogeneity of the studied cohort of CVID patients with various clinical manifestations, and patients were not analyzed for monogenic defects associated with CVID. In addition, assessment of the immunological profile in the peripheral blood may not represent the mechanisms generating and sustaining organ-specific chronic inflammation. Furthermore, treatment with low dose of glucocorticoids in some patients, might have had an influence on studied lymphocyte subsets.

Conclusions

Our findings indicate that the amount of $CD8^+$ T cells expressing PD-1 is associated with lymphadenopathy and number of regulatory T cells in patients with CVID. Additionally, patients with non-infectious, inflammatory complications have reduced number of peripheral regulatory T cells, depletion of naive $CD4^+$ T lymphocytes, and higher level of inflammation. Although the implication of our findings for pathogenesis of CVID associated noninfectious complications is not clear, it emphasizes the complexity of immune dysregulation associated with CVID. Additional studies are needed to verify whether regulatory T lymphocytes and PD-1 expression abnormalities contribute to the pathogenesis of CVID associated complications.

Conflict of interest

None declared.

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