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Phenotypic and Functional Analysis of T Follicular Cells in Common Variable Immunodeficiency

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Keywords
Follicular helper T cell · Follicular regulatory T cell · Common variable immunodeficiency · Autoimmune disease · Antibody

Abstract
Introduction: One of the most frequent abnormalities of B cells in common variable immunodeficiency (CVID) is reduced number of class-switched memory B cells, suggesting an impaired germinal center response. Therefore, due to its pivotal role in regulating the development of humoral immunity, the objective of this study was to evaluate the role of circulating T follicular helper (cT_{FH}) and circulating T follicular regulatory (cT_{FR}) cells in the pathogenesis of CVID. ***Methods:*** cT_{FH} and cT_{FR} cells from CVID patients and healthy subjects were phenotypically characterized by flow cytometry. cT_{FH} and memory B cells from CVID patients and healthy subjects were isolated and cocultured. ***Results:*** Our results showed a reduced proportion of $cT_{FH}17$ cells in patients with CVID and an increased ratio of cT_{FH}/cT_{FR} cells in CVID patients with autoimmune diseases. Furthermore, the proportion of IL-21-producing cT_{FH} cells was directly related to the proportion of $CD27^+$ IgD^- B cells. Interestingly, coculture assay showed that CVID-derived cT_{FH} cells are able to help memory B cells from healthy controls to produce immunoglobulins. ***Conclusions:*** The proportions of $cT_{FH}17$ and cT_{FR} cells are altered in CVID patients; however, the cT_{FH} function in assisting B cells to produce antibodies in vitro is preserved.

Introduction

Common variable immunodeficiency (CVID) is the most common symptomatic primary antibody deficiency disorder in adults characterized by decreased serum IgG and IgA or IgM associated with poor response to vaccines [1]. Although the majority of patients with CVID present with recurrent infections, especially of the upper and lower respiratory tract, approximately 20–30% develop autoimmune and inflammatory diseases [2–4]. Genetic mutations have been reported in CVID; however, they contribute to only 20% of the cases [5]. Therefore, pathogenic mechanisms in most of the CVID patients remain unknown.

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The analysis of B cell subsets in CVID patients showed a normal or reduced number of total B cells; however, in the majority of CVID patients, class-switched memory B cells are reduced, suggesting an impairment in activation and differentiation of B cells [2–4]. This reduction is also associated with clinical presentations such as splenomegaly, lymphadenopathy, and autoimmune diseases [2–4]. Some intrinsic defects in B cells that have been reported include impaired calcium influx after BCR activation, reduced activation after TLR stimulation, and increased susceptibility to apoptosis of memory B cells [5–7]. In contrast, some investigators have reported that B cells from a subset of CVID patients are able to produce in vitro normal levels of immunoglobulins, suggesting possible impairment in another event associated with germinal center (GC) reaction [8].

The germinal center formation is essential for B cell differentiation into memory cells and long-lived plasma cells to produce high-affinity antibodies. All of these events require a complex cooperation between follicular dendritic cells and $CD4^+$ T cells with B lymphocytes [9]. The ability of $CD4^+$ T cells to migrate to and support GC reaction depends on expression of high levels of chemokine receptor CXCR5 [10, 11]. These follicular helper T (T_{FH}) cells are additionally characterized by the expression of transcription factor B-cell lymphoma-6 (Bcl-6), programmed cell death receptor-1 (PD-1), inducible T-cell costimulator (ICOS), CD40 ligand (CD40L/CD154), and by their ability to produce IL-21 and IL-4 [10–12]. Signaling through those cytokines and cognitive interactions between T_{FH} and B cells support proliferation, survival, and antibody affinity maturation in B cells, as well as their differentiation into antibody-producing plasma cells and long-lived memory B cells [13, 14]. Besides T_{FH} cells, the GC formation is also regulated by follicular regulatory T (T_{FR}) cells that express FoxP3 protein and can control the immunological synapse between T_{FH} and B cells [13, 15].

It is known that both T_{FH} and T_{FR} cells can leave secondary follicles and contribute to the pool of circulating memory T cells [14, 16]. Phenotypically, circulating memory T_{FH} (cT_{FH}) cells, which are Bcl-6 negative, can be identified as $CD4^+CD45RA^-CXCR5^+$ T cells [14]. According to the expression of CXCR3 and CCR6 markers, cT_{FH} cells are further classified as $cT_{FH}1$ ($CXCR5^+CXCR3^+CCR6^-$), $cT_{FH}2$ ($CXCR5^+CXCR3^-CCR6^-$), and $cT_{FH}17$ ($CXCR5^+CXCR3^-CCR6^+$) cells. All of them are able to induce in vitro antibody production by memory B cells, but only $cT_{FH}2$ and $cT_{FH}17$ are able to help naïve B cells [14, 17]. Furthermore, the expression of ICOS and PD-1 has helped to identify more functional cT_{FH} cells [14, 17–19]. Although majority of cT_{FH} cells do not express ICOS or PD-1, a very small population of $ICOS^+PD\text{-}1^+$ T_{FH} cells is considered recently activated memory T_{FH} cells [19]. Circulating T_{FR} (cT_{FR}) cells also do not express Bcl-6, and the identification of $CD4^+CD45RA^-CXCR5^+FoxP3^+$ T cells in the peripheral blood indicates GC activity [16].

In the last decade, understanding of the biology of T_{FH} and T_{FR} cells and their contribution to disease states has significantly increased [20]. Several studies have demonstrated a positive correlation between the proportion of different subtypes of cT_{FH} cells and the production of neutralizing IgG antibodies against certain viruses [21, 22]. Additionally, disturbances in the cT_{FH} and cT_{FR} cell compartment are associated with development and severity of autoimmune diseases [14, 23, 24]. Previous studies have shown differences in the frequency of cT_{FH} cells between CVID patients and healthy subjects [25–28]. However, no studies have examined their functions in antibody production by B cells. Therefore, we have performed both phenotypic and functional analyses of cT_{FH} cells and phenotypic analysis of cT_{FR} cells to understand a probable involvement of these cells in the CVID pathogenesis.

Material and Methods

Patients

Peripheral venous blood samples were obtained from 32 patients (18 female and 14 male) diagnosed with CVID. Pan-American Group for Immunodeficiency and European Society for Immunodeficiencies (ESID) criteria were used to diagnose CVID patients [29]. Blood samples were drawn just prior to immunoglobulin administration (at trough level). Blood samples from 29 age- and sex-matched healthy subjects were used as controls. The protocol was approved by the Institution Review Board of the University of California, Irvine, and an informed consent was signed by each subject.

Flow Cytometry Analysis

Peripheral blood mononuclear cells (PBMC), separated by density gradient centrifugation with Lymphocyte Separation Medium (Life Technology, Grand Island, NY, USA), were resuspend with the RPMI medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 1×10^6 cells/mL and then incubated with various combinations of monoclonal antibodies and isotype controls. The following antibodies were used to identify different T cells and B cell subsets: anti-CD4-PerCP, anti-CD45RA-BV510, anti-CXCR5-Alexa Fluor 488, anti-CCR6-PE, anti-CXCR3-BV421, anti-PD-1-PE-CF594, anti-IgM-APC, anti-IL-21R-PE-CF594 (all from BD Bioscience, San Diego, CA, USA), anti-ICOS-BV786, anti-CD19-PerCP, anti-CD27-FITC, anti-IgD-BV510, and anti-CD38-PE (from BioLegend, San Diego, CA, USA).

Table 1. Characteristics of CVID patients and control subjects

	Control	CVID patients	
		no autoimmune disease	with autoimmune disease
Subjects, *n*	29	20	12
Mean age, years	47.8	53.7	52.9
Sex (female/male)	17/12	10/10	8/4
Splenomegaly	N/A	0	0
Autoimmune manifestation	N/A	N/A	
Autoimmune thrombocytopenia			4
Hypothyroidism			4
Rheumatoid arthritis			2
ANCA vasculitis			1
Adrenal insufficiency			1
Treatment	N/A		
Intravenous		11	5
Subcutaneous		4	6
Lymphocyte subset			
$CD4^+$ T cell, cell/mm^3 (mean)[1]	N/A	662.9	643.8
$CD8^+$ T cell, cell/mm^3 (mean)[1]	N/A	305.4	574.8
$CD19^+$ B cell, cell/mm^3 (mean)[1]	N/A	183.5	196.6
$CD19^+$ cells, %[2]	5.6	5.0	6.6
$CD27^+IgD^-$, % of $CD19^+$ cells (mean)[2]	16.0	9.5*	8.9*

CVID, common variable immunodeficiency. [1] Values obtained from medical records. Normal ranges of lymphocyte subset: $CD4^+$ T cell (338–1,194), $CD8^+$ T cell (85–729), and $CD19^+$ B cell (51–473). [2] Values obtained from sample blood at the time of recruitment. * p value = 0.0083 compared with the control group.

For cytokine measurement, PBMC was stimulated in 24-well flat bottom plates with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL; Sigma-Aldrich, Saint Louis, MO, USA) plus ionomycin (IO, 1 μg/mL; Sigma-Aldrich, Saint Louis, MO, USA) and brefeldin A (BD Pharmingen, San Diego, CA, USA) at 37°C in a humidified 5% CO_2 incubator for overnight. In brief, the cells were incubated with various combinations of mAbs for surface markers for 20 min at room temperature in the dark, according to the manufacturer's instructions. The cells were washed with wash solution and then permeabilized by incubating cells with Cytofix/Cytoperm solution (BD Pharmingen, San Diego, CA, USA) at 4°C for 20 min. After washing, the mAbs for intracellular staining (anti-IL-21-BV786, IFN-γ-BV786, anti-IL-4-BV786, and anti-IL-17-BV786 from BD Biosciences, San Diego, CA, USA) were added and incubated for 30 min at 4°C.

For FoxP3 staining, fresh PBMC was incubated with anti-CD4-PerCP, anti-CD45RA-BV510, anti-CXCR5-Alexa Fluor 488, anti-CD25-BV421, and anti-PD-1-PE-CF594 for 20 min at room temperature for surface staining. The cells were fixated and permeabilized using the Human FoxP3 Buffer Set from BD Pharmingen™ (San Diego, CA, USA), according to the manufacturer's instructions. After washing, the cells were stained with anti-FoxP3-PE for 30 min at room temperature.

The cells were acquired using FACSCelesta (Becton-Dickinson, San Jose, CA, USA) and analyzed using Flow Jo software. Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. After acquisition of 100,000–200,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells and doublets.

Cell Sorting and Coculture Assay

After PBMC isolation, $CD4^+$ T cells and B cells were enriched by negative and positive selection, respectively, using magnetic columns according to the manufacturer's instructions (EasySep™, StemCell Technology, Vancouver, BC, Canada). The $CD4^+$ T cells were stained with anti-CD4-PerCP-Cy5.5, anti-CD45RA-APC, and anti-CXCR5-Alexa Fluor 488, and the B cells were stained with anti-CD19-PerCP-Cy5.5 and anti-CD27-PE (all antibodies from BD Biosciences, San Diego, CA, USA). The T_{FH} cells, defined as $CD4^+CD45RA^-CXCR5^+$, and memory B cells, defined as $CD19^+CD27^+$, were sorted using an FACSAria II cell sorter (Becton-Dickinson, San Jose, CA, USA).

T_{FH} cells (1×10^5 cell/mL) were cultured with memory B cells (2×10^5 cell/mL) in the AIM-V serum-free medium in the presence of Dynabeads® Human T-activator CD3/CD28 beads (Life Technologies, Carlsbad, CA, USA) and CpG-ODN2006 (InvivoGen, San Diego, CA, USA) (2.5 μg/mL) in a 96-well U bottom plate at 37°C in a humidified 5% CO_2 incubator. As a control, memory B cells were also cultured alone without any stimulus or with CpG-ODN (2.5 μg/mL). After 7 days, the supernatants were collected. The T_{FH} cells were cultured alone with or without anti-CD3 and anti-CD28 beads. After 3 days, the supernatants were collected.

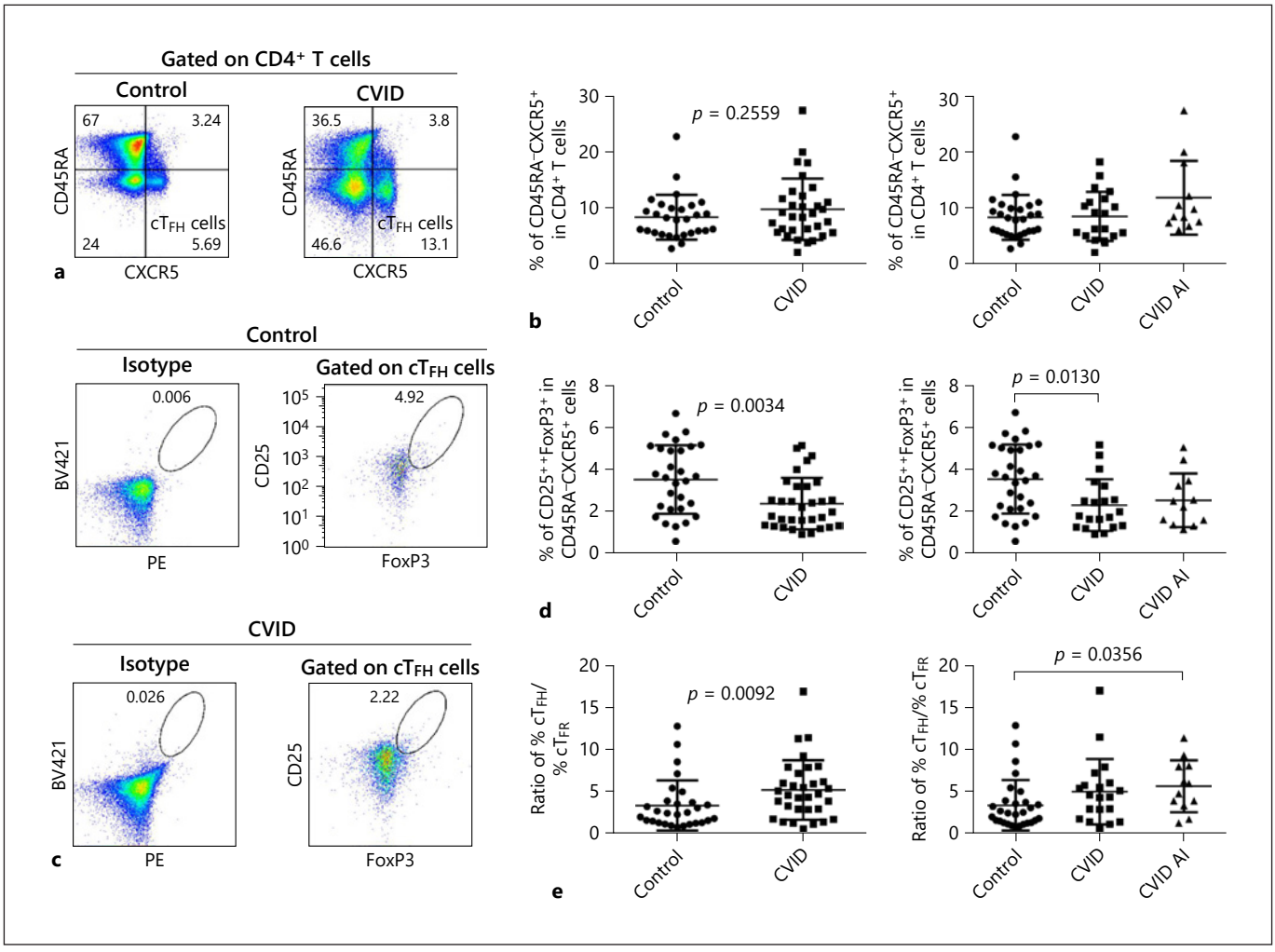

Fig. 1. The percentage of cT_{FH} and cT_{FR} cells in CVID patients. PBMC from CVID patients and healthy subjects were isolated and stained with specific monoclonal antibodies to identify cT_{FH} cells. **a** Representative dot-plots of cT_{FH} cells ($CD4^+CD45RA^-CXCR5^+$). **b** The cT_{FH} cells were analyzed in healthy subjects (control) and CVID patients with (CVID AI) or without autoimmune disease (CVID). **c** Representative dot-plots of cT_{FR} cells ($CD4^+CD45RA^-CXCR5^+FoxP3^+CD25^{++}$). **d**, **e** The cT_{FR} cells and cT_{FH}/cT_{FR} ratio were analyzed in healthy subjects (control) and CVID patients with (CVID AI) or without autoimmune disease (CVID). The mean values were compared, and the significant *p* values are shown. cT_{FH}, circulating T follicular helper; cT_{FR}, circulating T follicular regulatory; CVID, common variable immunodeficiency; PBMC, peripheral blood mononuclear cell.

Measurement of Immunoglobulins and Cytokines

The concentrations of IgG, IgA, and IgM from cocultures were determined by using the ELISA kit from ThermoFisher Scientific (Life Technologies, Carlsbad, CA, USA). The plates were read at 450 nm in an ELISA reader (Dynex Technologies, Chantilly, VA, USA). The results were interpolated from the standard curve constructed by using the standard provided with the kits. The detection limit of IgG, IgA, and IgM was 1.6, 1.6, and 15.6 ng/mL, respectively. The cytokines IL-4, IL-10, IL-21, and IFN-γ from T_{FH} cell cultures were analyzed by multiplex assay (ThermoFisher, Life Technologies, Carlsbad, CA, USA).

Statistical Analysis

All statistical analyses of the tests were conducted using the program GraphPad Prism graphic version 6.0 for Windows. The nonparametric Mann-Whitney U test or Student's *t* test were applied to determine whether the 2 groups were statistically different for each given variable. To compare more than 2 groups, we used one-way ANOVA followed by the Tukey test for data with Gaussian distribution and the Kruskal-Wallis followed by Dunn's test for data without Gaussian distribution. Correlations between variables were ascertained using Pearson's or Spearman's correlation. The significance in all experiments was defined as $p < 0.05$.

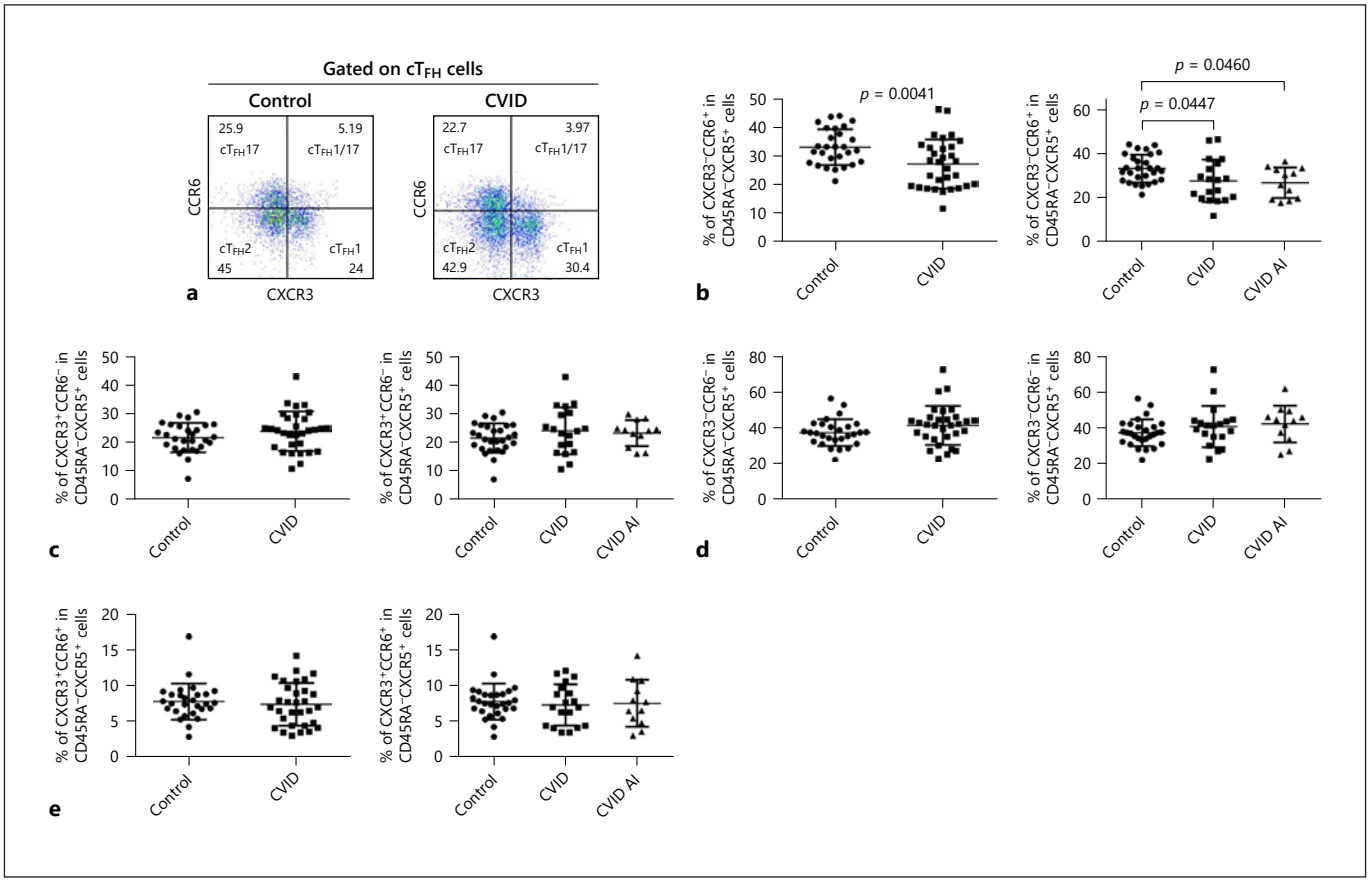

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Fig. 2. The percentage of different subsets of cT_{FH} cells in CVID patients. PBMC from CVID patients and healthy subjects were isolated and stained with specific monoclonal antibodies to identify cT_{FH} cells. **a** Representative dot-plots of cT_{FH} subsets, $cT_{FH}17$ ($CXCR5^+CXCR3^-CCR6^+$), $cT_{FH}1$ ($CXCR5^+CXCR3^+CCR6^-$), $cT_{FH}2$ ($CXCR5^+CXCR3^-CCR6^-$), and $cT_{FH}1/17$ ($CXCR5^+CXCR3^+CCR6^+$). **b–e** The cT_{FH} subsets were analyzed in healthy subjects (control) and CVID patients with (CVID AI) or without autoimmune disease (CVID). The mean values were compared, and the significant *p* values are shown. cT_{FH}, circulating T follicular helper; CVID, common variable immunodeficiency; PBMC, peripheral blood mononuclear cell.

Results

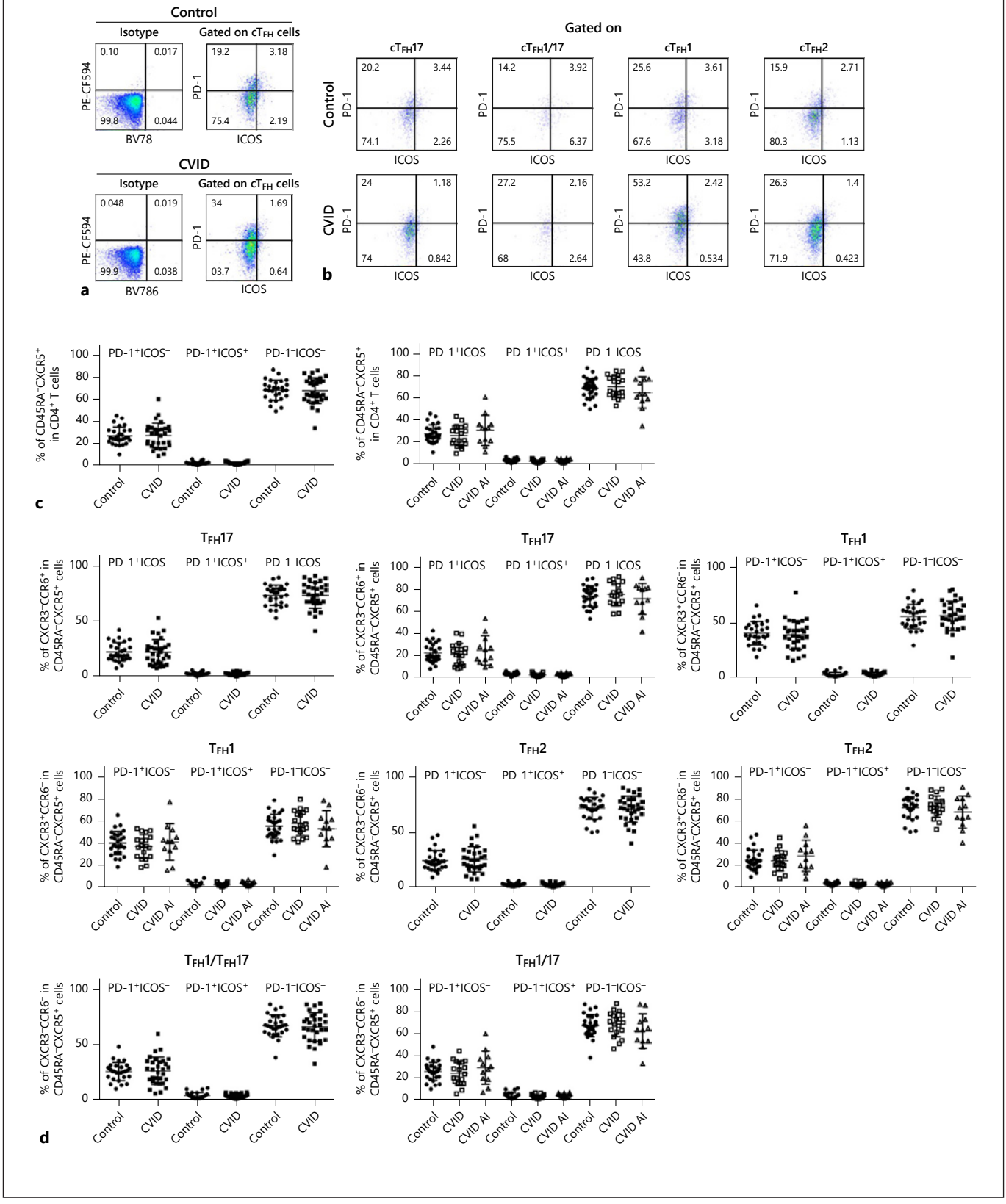
Characteristics of CVID Patients

Characteristics of CVID patients and healthy controls are shown in Table 1. Twelve patients were diagnosed with autoimmune diseases. None of the patients with autoimmune diseases were on immunosuppressive agents. The majority of patients ($n = 30$) were receiving intravenous or subcutaneous immunoglobulin replacement therapy. In regard to B cell percentages, 2 patients (1 with autoimmune disease) had less than 1% and 27 patients (11 with autoimmune disease) had more than 1% of total $CD19^+$ cells. Between the patients with more than 1% of total $CD19^+$ cells, 4 patients (2 with autoimmune disease) had less than 2% of $CD27^+IgD^-$ B cells. The information about B cell from 3 patients was not available.

CVID Patients Showed Reduced Percentage of $cT_{FH}17$ and cT_{FR} Cells

The immunophenotyping analysis of $CD4^+$ T cells was performed, and memory cT_{FH} cells, defined as $CD4^+CD45RA^-CXCR5^+$, and cT_{FR} cells, defined as $CD4^+CD45RA^-CXCR5^+FoxP3^+CD25^{++}$, were evaluated (Fig. 1a, c). No difference was observed in the percentage of cT_{FH} cells between the control group and total CVID patients and patients with or without autoimmune diseases (Fig. 1b). In contrast, CVID patients showed decreased proportion of cT_{FR} cells (Fig. 1d). Also, an increased cT_{FH}/cT_{FR} ratio, mainly in the patients with autoimmune disease, was observed in patients as compared with the control group (Fig. 1e).

Identification of different cT_{FH} subsets by CXCR3 and CCR6 expression was also performed (Fig. 2a), and



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the percentage of $cT_{FH}17$ ($CXCR5^+CXCR3^-CCR6^+$) was significantly lower in CVID patients, with or without autoimmune disease, as compared with the control subjects (Fig. 2b). No difference was found in the percentage of $cT_{FH}1$ ($CXCR5^+CXCR3^+CCR6^-$), $cT_{FH}2$ ($CXCR5^+CXCR3^-CCR6^-$), and $cT_{FH}1/17$ ($CXCR5^+CXCR3^+CCR6^+$) subsets between healthy subjects and CVID patients (Fig. 2c–e).

Table 2. Percentage of IL-21R on B cells

	Control	CVID	*p* value
% IL-21R in $CD19^+$ cells (mean)	1.46	1.46	0.9772
% IL-21R in $CD19^+CD27^-$ cells (mean)	1.66	1.36	0.4357
% IL-21R in $CD19^+CD27^+$ cells (mean)	0.66	1.73	**0.0203**

PBMC from CVID patients and healthy subjects were isolated and stained with specific monoclonal antibodies to analyze the expression of IL-21R on B cells. The mean values were compared, and the significant *p* values are shown in bold.

Table 3. Expression of PD-1, ICOS, and CD40L and cytokine production in cT_{FH} cells after stimulation in CVID patients

	Control	CVID	*p* value
% PD-1 in $CXCR5^+CD4^+$ T cells	57.3	32.8	0.3929
% ICOS in $CXCR5^+CD4^+$ T cells	57.9	33.5	0.3929
% CD40L in $CXCR5^+CD4^+$ T cells	1.4	2.3	0.6786
IL-4, pg/mL	44.8	148.5	>0.9999
IL-10, pg/mL	87.9	105.0	0.7857
IFN-γ, pg/mL	370.1	286.3	0.7857
IL-21, pg/mL	38.0	37.6	0.8571

Sorted cT_{FH} cells from CVID patients ($n = 6$) and the control group ($n = 3$) were stimulated with anti-CD3/CD28 beads for 3 days. The cytokines were measured by multiplex assay, and the expression of costimulatory molecules was analyzed by flow cytometry. The mean values were compared, and the *p* values are shown. PD-1, programmed cell death receptor-1; ICOS, inducible T-cell costimulator; cT_{FH}, circulating T follicular helper; CVID, common variable immunodeficiency; CD40L, CD40 ligand.

Fig. 3. PD-1 and ICOS expression on cT_{FH} cells and their subsets in CVID patients. PBMC from CVID patients and healthy subjects were isolated and stained with specific monoclonal antibodies to identify cT_{FH} cells. **a**, **b** Representative dot-plots of PD-1 and ICOS expression on cT_{FH} cells and their subsets, respectively. **c** PD-1 and ICOS expression were analyzed in cT_{FH} cells from healthy subjects (control) and CVID patients with (CVID AI) or without autoimmune disease (CVID). **d** PD-1 and ICOS expression were analyzed on $cT_{FH}17$, $cT_{FH}1$, $cT_{FH}2$, and $cT_{FH}1/17$ subsets in the control group and in CVID patients with (CVID AI) or without autoimmune disease (CVID). PD-1, programmed cell death receptor-1; ICOS, inducible T-cell costimulator; cT_{FH}, circulating T follicular helper; CVID, common variable immunodeficiency; PBMC, peripheral blood mononuclear cell.

PD-1 and ICOS Expression on cT_{FH} Cells from CVID Patients

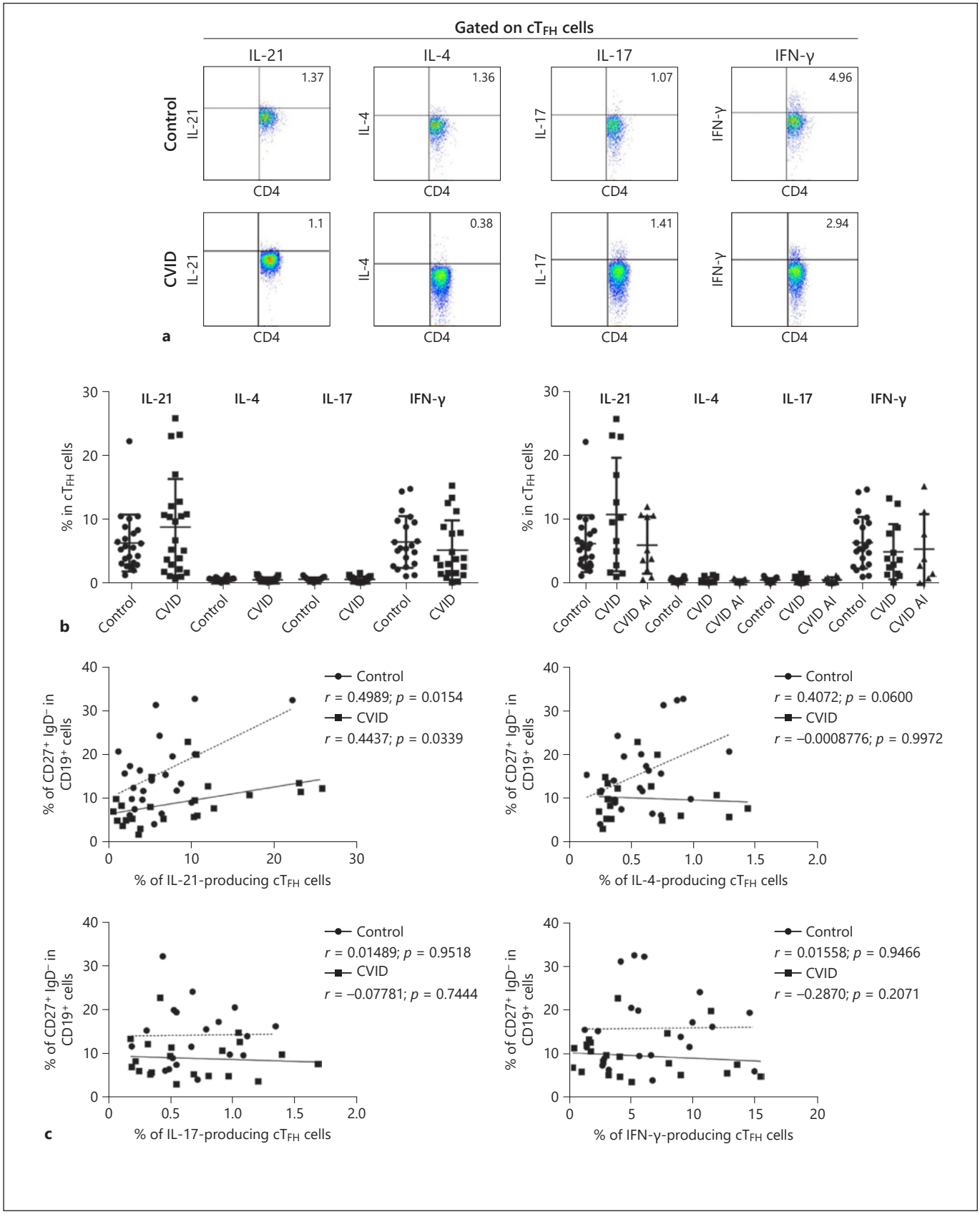
The PD-1 and ICOS expression has facilitated the identification of better functional cT_{FH} cells [23]. As shown in Figure 3c, most of the cT_{FH} cells do not express both ICOS and PD-1 and a very small frequency of these cells are $ICOS^+PD\text{-}1^+$ or $ICOS^+PD\text{-}1^-$. Taking into account these markers, no difference was observed with regard to the frequency of $ICOS^+PD\text{-}1^+$ and $ICOS^-PD\text{-}1^+$ cT_{FH} cells between the control and CVID patients (Fig. 3c). Besides the low percentage of $cT_{FH}17$ subset in CVID patients, we did not observe difference in PD-1 and ICOS expression on the cT_{FH} cell subset (Fig. 3d).

IL-21-Producing cT_{FH} Cells Are Associated with Class-Switched Memory B Cells

The cytokine production by T_{FH} cells, especially IL-21, provides signals for B cells to proliferate and differentiate into antibody-producing cells. No significant difference was observed between CVID and control groups regarding to the percentage of IL-21, IL-4, IL-17, and IFN-γ-producing cT_{FH} cells (Fig. 4b). Interestingly, we observed a positive correlation between the percentage of IL-21-producing cT_{FH} cells and the proportion of class-switched memory B cells in both CVID patients and the control group, although the percentage of those cells was similar in CVID patients and healthy subjects (Fig. 4c). Since the percentage of cytokine-producing cT_{FH} cells was not impaired in CVID patients, we analyzed the expression of IL-21 receptor (IL-21R) on B cells of these patients. The percentage of total B and naïve B cells, defined as $CD27^-$, expressing IL-21R was not significantly different between CVID patients and controls. In contrast, CVID patients showed higher percentage of $CD19^+CD27^+$ $IL\text{-}21R^+$ cells compared with the control group (Table 2).

cT_{FH} Cells from CVID Patients Are Able to Help the Antibody Production in vitro

We further evaluated the functional capacity of cT_{FH} cells to help B cells in vitro. We observed that memory B cells from a subset of CVID patients, named as CVID+, produced levels of immunoglobulin comparable to the control group (Fig. 5a). However, B cells from another subset of CVID patients, named as CVID–, even in the



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presence of cT_{FH} cells, were unable to produce any detectable levels of IgG and IgA when activated with CpC-ODN. On the other hand, IgM production was similar between those patients and controls. To verify if the impaired immunoglobulin production in CVID− patients was due to inability of cT_{FH} cells to help B cells, we cocultured cT_{FH} cells from CVID− patients with B cells from control groups. Also, cT_{FH} cells from healthy individuals were cocultured with B cells from CVID− patients. As observed in Figure 5b, the production of IgG and IgA by CVID− B cells was not recovered after coculturing with cT_{FH} cells from the control group; however, IgG and IgA production in the coculture with cT_{FH} cells from CVID− patients and B cells from healthy subjects was similar to the cocultures with autologous cells from the control group. The cT_{FH} cells were also activated with anti-CD3/anti-CD28 beads for 3 days to verify the expression of CD40L, PD-1, and ICOS and the production of IL-21, IL-4, IL-10, and IFN-γ. As shown in Table 3, no difference was observed concerning the expression of CD40L, PD-1, and ICOS and the production of IL-21, IL-4, IL-10, and IFN-γ by T_{FH} cells.

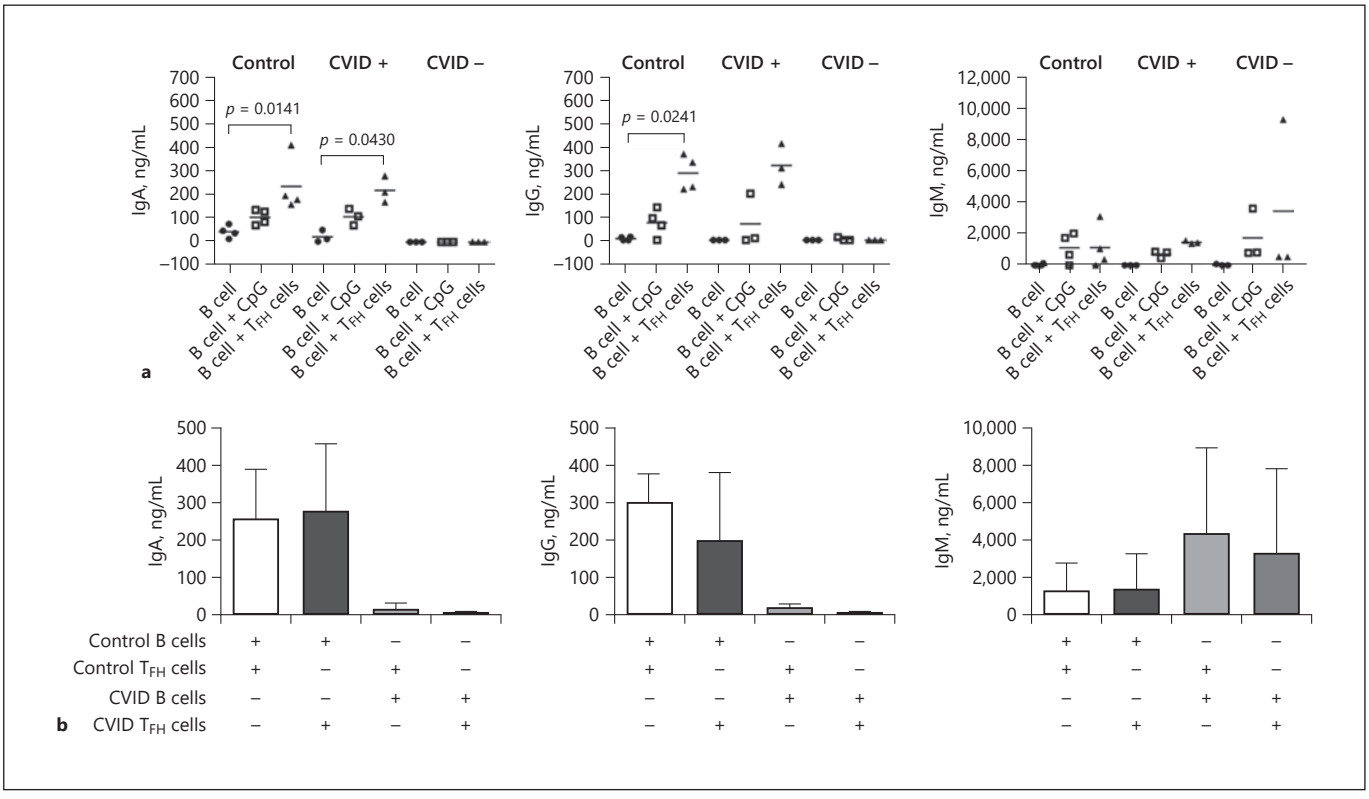

Fig. 5. Immunoglobulin production by cT_{FH}/B cells cocultures from CVID patients. **a** cT_{FH} cells and memory B cell from CVID patients ($n = 6$) and the control group ($n = 4$) were cocultured for 7 days, and IgA, IgG, and IgM production were dosed in the supernatants by ELISA. **b** T_{FH} cells from CVID− patients ($n = 3$) and B cells from the control group or T_{FH} cells from the control group and B cells from CVID− patients ($n = 3$) were cocultured for 7 days for immunoglobulin detection. The mean values were compared, and the *p* values are presented. cT_{FH}, circulating T follicular helper; CVID, common variable immunodeficiency.

Fig. 4. Cytokine production by cT_{FH} cells in CVID patients. PBMC from CVID patients and healthy subjects were isolated, incubated with PMA, ionomicyn, and brefeldin A overnight, and stained with specific monoclonal antibodies to identify cytokine-producing cT_{FH} cells. **a** Representative dot-plots of cytokine-producing cT_{FH} cells. **b** The percentage of cT_{FH} cells able to produce IL-21, IL-4, IL-17, and IFN-γ in the control group and in CVID patients with (CVID AI) or without autoimmune disease (CVID). **c** Correlation of percentage of cytokine-producing-cT_{FH} cells and the percentage of class-switched memory B cells in CVID patients. The mean values were compared, and the *p* values are shown. cT_{FH}, circulating T follicular helper; CVID, common variable immunodeficiency; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate.

Discussion

Patients with CVID present low levels of serum IgG and IgA or IgM, poor response to vaccines, and are more susceptible to infections and immune dysregulation [1, 30]. The reduced numbers of class-switched memory B cells is a characteristic of these patients, suggesting a defect during GC reaction [2–4]. Due to its pivotal role in controlling efficient antibody production, disturbances in the T_{FH} cell compartment have been related to the immunopathogenesis of various human diseases, such as infections, autoimmunity, and primary immunodeficiencies [14, 21, 28].

Recent studies have identified increased levels of cT_{FH} cells and $cT_{FH}1$ subset in CVID patients with one or more complications, such as splenomegaly, lymphadenopathy, enteropathy, and granulomatous and autoimmune disease, when compared with patients without these clinical complications and controls [26–28]. Additionally, in one study, most of the patients had less than 2% of $CD27^+IgD^-$ B cells [27], which differs from our cohort. However, in other studies, the patients were not divided according to the number of $CD27^+IgD^-$ B cells [26, 28], and the reduced number of class-switched memory B cells is associated with worst clinical presentation [2–4]. In the present study, we did not observe similar alterations in cT_{FH} cells or $cT_{FH}1$ subset, which could be explained, at least in part, due to absence of splenomegaly and other complications, besides autoimmunity, in our patients associated with a percentage higher than 2% of $CD27^+IgD^-$ B cells in the majority of our cohort. In agreement with our results, Cunill et al. [25] observed increased percentages of cT_{FH} and $cT_{FH}1$ cells only in CVID patients with less than 2% of $CD27^+IgD^-$ B cells and no significant changes in patients with more than 2% of $CD27^+IgD^-$ B cells.

Regarding $cT_{FH}17$ and $cT_{FH}2$ subsets, both are memory cells able to induce in vitro naïve and memory B cells to produce IgG and IgA or IgE, respectively, and the increase in these subsets has been associated with the expansion of blood plasmablasts and autoantibody levels in some autoimmune diseases [23, 24]. Our results showed lower percentage of $cT_{FH}17$ cells in CIVD patients with or without autoimmune diseases, and no difference in the percentage of $cT_{FH}2$ cells was found. Previous studies observed normal or reduced frequencies of $cT_{FH}17$ and $cT_{FH}2$ subsets in CVID patients [25–27]. Interestingly, the reduced frequencies of these subsets were in CVID patients with inflammatory complications [26, 27]. Here, the lower proportion of $cT_{FH}17$ cells in patients without autoimmune diseases differs from previous studies but may help to explain, at least in part, the low levels of IgG and IgA. Taken together, the distinct results in the percentages of cT_{FH} subsets we found, compared with previous studies, may be explained, in part, by the differences in our patient cohort, composed of patients with less inflammatory complications and higher percentage of $CD27^+IgD^-$ B cells.

Autoimmune diseases are common manifestations among patients with primary immunodeficiencies [31]. For this reason, we stratified CVID patients according to the presence or absence of autoimmune manifestations. In our cohort, the most prevalent autoimmune diseases were autoimmune thrombocytopenia, hypothyroidism, and rheumatoid arthritis. Interestingly, these are autoantibody-mediated autoimmune diseases. Furthermore, studies have showed both an expansion of cT_{FH} cells and an association with the autoantibody titers in these autoimmune diseases [32–34]. In the present study, CVID patients with autoimmune diseases showed increased, but not significant, proportion of cT_{FH} cells. Moreover, we observed that CVID patients had low percentage of cT_{FR} cells compared with the control group. Indeed, studies have reported an association between T_{FR} cell deficiency and the development of autoimmunity in mice [35]. We also observed decreased percentage of cT_{FR} cells in CVID patients, as well as an increase in the cT_{FH}/cT_{FR} ratio, only in CVID patients with autoimmune diseases. Interestingly, Romberg et al. [36] observed increased number of T_{FH} cells and a decrease of Treg-cell frequency in GC of CVID patients with autoimmune cytopenias as compared with both CVID patients without autoimmune disease and healthy individuals. Furthermore, Cunill et al. [25] observed a reduction in cT_{FR} cells from CVID patients with less than 2% of $CD27^+IgD^-$ B cells. The increase in the cT_{FH}/cT_{FR} ratio in patients with autoimmune disease may suggest an imbalance between cT_{FH} and cT_{FR} cells that contribute to an impaired regulation of GC reaction leading to the loss of tolerance.

The cytokines produced by T_{FH} cells provide signals for B cells to proliferate, survive, and differentiate. Besides IL-21, the signature cytokine of T_{FH} cells, subsets of cT_{FH} cells are also able to secrete IFN-γ, IL-4, and IL-17 [13]. Reduced production of IL-17 by $CD4^+$ T cells has been associated with reduced number of $CD27^+IgD^-$ B cells in CVID patients and healthy subjects [37, 38]. Moreover, stimulation of PBMC from CVID patients with IL-4 and IL-21 prevented apoptosis of B cells and enhanced immunoglobulin production [39]. In the present study, we did not observe any significant difference in cytokine production by cT_{FH} cells from CVID patients,

but we did find a positive correlation between the percentage of IL-21-producing cT_{FH} cells and the frequency of class-switched memory B cells in CVID patients and healthy control. Loss-of-function mutation in the IL-21R gene causes immunodeficiencies in human, and IL-21 receptor-deficient mice showed diminished serum levels of IgG1, IgG2, and IgG3 associated with impaired memory B cell response [40, 41]. Therefore, we evaluated the percentage of B cells expressing IL-21R. Interestingly, the percentage of $CD27^+$ B cells expressing IL-21R was increased in CVID patients compared with the control group that may suggest a compensatory mechanism.

In addition to cytokine production, the cognitive interactions between T_{FH} cells and B cells are also essential for GC reaction and differentiation of B cells into memory B cells and long-lived high-affinity plasma cells. In this context, PD-1 and ICOS are important molecules for T_{FH} cell function and are highly expressed in the GC [42, 43]. Therefore, the presence of $ICOS^+PD\text{-}1^+$ cT_{FH} cells indicates ongoing GC reaction [19]. Here, we did not find difference in the percentage of $PD\text{-}1^+ICOS^+$, $PD\text{-}1^+ICOS^-$, and $PD\text{-}1^-ICOS^-$ cells in neither total cT_{FH} cells nor in their subsets between the patients and control group. In contrast, although they did not evaluate ICOS expression, Cunill et al. [25] observed a high frequency of $PD\text{-}1^+$ cT_{FH} cells in CVID patients with less than 2% of $CD27^+IgD^-$ B cells and Coraglia et al. [28] observed a high frequency of $PD\text{-}1^+$ cT_{FH} cells in CVID patients with autoimmune disease and/or granulomatous disease. The discordance observed between our results may be explained, at least in part, by the clinical differences in our cohorts.

Besides the decreased percentage of $cT_{FH}17$ cells in CVID patients, we did not observe differences in cytokine production and in the expression of PD-1 and ICOS molecules between CVID patients and controls. Therefore, we cocultured cT_{FH} cells with memory B cells to evaluate the immunoglobulin production. Our analysis showed 2 groups of CVID patients according to IgG and IgA production in vitro. In a subset of patients, called as CVID+, memory B cells were able to produce similar levels of IgG and IgA when compared with the control group. However, in other subset of patients, called as CVID−, memory B cells were incapable to produce any detectable levels of IgG and IgA. In agreement with our results, some previous studies also observed in vitro that B cells or PBMC from some CVID patients have normal immunoglobulin production, whereas other studies have not confirmed it [8, 44].

Experiments with allogeneic cocultures showed that cT_{FH} cells from the CVID− group were capable to help B cells from healthy subjects. In contrast, B cells from CVID− patients failed to produce immunoglobulins even when cocultured with cT_{FH} cells from healthy individuals. We also cocultured cT_{FH} cells from the CVID + group with B cells from healthy control, and the levels of IgG and IgA were similar to the autologous coculture (data not shown). Thus, our results suggest that cT_{FH} cells from CVID− and CVID+ patients have preserved functions. Indeed, we did not observe any difference concerning the production of IL-21, IL-4, and IFN-γ by T_{FH} cells, as well as the expression of CD40L, PD-1, and ICOS, following activation of cT_{FH} cells from CVID patients with anti-CD3 and anti-CD28 beads. In agreement with these results, a study published by Coraglia et al. [28] also showed that, in PBMC activated with PHA and IL-2, cT_{FH} cells from CVID patients were able to respond by upregulating CD40L and ICOS. These results suggest a defect in B cells from CVID patients as the mechanism for impaired immunoglobulin production. This may be due to the impaired signaling pathway on B cells. In this context, Clemente et al. [45] showed that memory B cells from some CVID patients have increased STAT3 activation that could increase the susceptibility to apoptosis and contribute to the low levels of immunoglobulins.

Interestingly, when we evaluate the cT_{FH} cell phenotype in CVID patients, we did not observe differences in the frequency of cT_{FH} and cT_{FR} cells between the CVID groups (CVID− and CVID+) and control (data not shown). About the cT_{FH} subsets, the CVID− patients showed lower frequency of $cT_{FH}17$ cells than control and a lower frequency of class-switched memory B cells compared with CVID+ and control groups (data not shown). However, our cT_{FH} cell functional analysis that was conducted by evaluating the cytokine production, the expression of costimulatory molecules, and the help to B cells in vitro showed no differences between CVID patients and the control group. Together, these results may suggest that the immunophenotyping of cT_{FH} cells by chemokine receptors is not the best method to identify functional cT_{FH} cells in CVID patients. Increasing the number of subjects in the coculture experiments will help us to determine if there is a correlation between the lower frequency of $cT_{FH}17$ cells and class-switched memory B cells with the lower levels of IgG and IgA in these patients.

In conclusion, our results show that the proportions of $cT_{FH}17$ and cT_{FR} cells are altered in CVID patients; however, their function in assisting B cells to produce antibodies in vitro is preserved. This would suggest an intrinsic defect in B cells in CVID as a mechanism for impaired immunoglobulin production.

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Statement of Ethics

The protocol was approved by the Institution Review Board of the University of California, Irvine, and an informed consent was signed by each subject.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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Author Contributions

T.K. performed the experiments, analyzed the data, and wrote the manuscript. C.B. provided critical review and wrote the manuscript. S.G. designed the experiments, coordinated the recruitment of patients, and wrote the manuscript.

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