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ORIGINAL ARTICLE

Lymphocytes and B-cell abnormalities in patients with common variable immunodeficiency (CVID)

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Abstract
Background and aims: Common variable immunodeficiency (CVID) is a primary antibody deficiency characterised by decreased antibody production and low or normal B-cell numbers. To elucidate the clinical and immunological heterogeneity of CVID, we studied 16 patients diagnosed with CVID.
Methods: We analysed B, T and NK cell populations. We also assessed CD27 expression to define B-cell subsets and examined the expression of molecules important in B-cell proliferation and differentiation, such as the transmembrane activator and CALM interactor (TACI), inducible costimulator (ICOS), CD154 and CD40.
Results: We observed reduced B and T-cell numbers in CVID patients; this reduction was more pronounced in adults. While one group of patients (group I) showed a significant reduction in CD27+ memory B-cells, another group (group II) of patients exhibited numbers of CD27+ memory B-cells similar to the healthy donor. The frequency of B-cells and T-cells expressing CD40 and ICOS, respectively, was significantly lower in all CVID patients compared with healthy donors. Finally, a correlation between the frequency of CD27+ memory B-cells and clinical features was observed in CVID patients.
Conclusion: These results suggest that in some patients, the combined defects in both T and B-cells may account for CVID. Additionally, patients in group I exhibited an increased frequency of pneumonia and chronic diarrhoea.

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Introduction

After selective IgA deficiency, common variable immunodeficiency (CVID) is the most common antibody-based primary immunodeficiency. CVID patients have decreased levels of IgG and IgA and, in some cases, IgM. Patients suffer from recurrent infections, predominantly of the respiratory and gastrointestinal tracts. CVID is also associated with autoimmune, inflammatory and lymphoproliferative manifestations and malignancies.[1,2] The number of peripheral B-cells are normal in most cases.[3] Several cellular defects have been observed in patients with CVID, including alterations in the proliferation and activation of T-cells, irregular cytokine production and decreased expression of costimulatory molecules such as CD40L.[4] However, the most noticeable defects occur in B-cells, particularly during B-cell differentiation affecting the processes of somatic hypermutation and isotype switching.[5] Additionally, a small number of CVID patients have mutations in *CD19*, *TNFRSF13B* (TACI), *TNFRSF13C* (BAFF-R), inducible costimulator (ICOS), *CD20*, *CD81*, *CD21* and *MSH5*.[6–13]

Several laboratories have proposed the classification of CVID patients based on memory B-cell numbers. Further, enumeration of memory B-cells in CVID has been proposed as a prognostic marker for respiratory disease, autoimmunity and granulomatous disease.[14,15]

To date, no studies of CVID patients in Mexico have been performed. For this reason, we characterised multiple immunological parameters in 16 patients diagnosed with CVID. Lymphocyte populations and the expression of molecules important for B-cell proliferation and differentiation, such as TACI, ICOS, CD154 and CD40 were analysed. Additionally, the presence of memory B-cells was assessed using CD27 expression as a marker. Thus, the main purpose of this work was to identify potential abnormalities in the expression of these molecules and to correlate any documented abnormalities with clinical features with the aim to better characterise CVID and contribute to the understanding of this pathology.

Material and methods

Patients and controls

In total, 16 CVID patients from the Instituto Nacional de Pediatría and Centro Médico Nacional ''La Raza'' Instituto Mexicano del Seguro Social, Mexico City, were included in the study. All patients fulfilled the criteria for CVID based on definitions from the European Society for Immunodeficiency (ESID) and the Pan-American Group for Immunodeficiency (PAGID); these definitions stipulated a marked decreased of IgG at least two standard deviations (SDs) below the age-matched mean, reduced serum IgA and/or IgM, age of onset greater than two years and exclusion of other causes of hypogammaglobulinaemia.[16]

In this work, all participating patients displayed reduced levels of at least two Ig isotypes and IgG serum levels below 600 mg/dL. Clinical information was compiled from each subject from medical files at the time of the study, all these manifestations are included in Table 1. This study was performed with the consent of all patients and the corresponding institutions and the study received approval from the ethics committee from each participating institution. We also included 64 controls aged 5–50 years (mean, 19 years) for phenotypic analysis. The control group comprised of 24 males and 40 females.

Enumeration of lymphocyte subpopulations

To assess the different lymphocyte subpopulations, we performed flow cytometric analysis in controls and CVID patients prior to intravenous gammaglobulin (IVIG) infusion. Lymphocyte populations were enumerated in whole-blood samples and stained with the following mixtures of monoclonal antibodies (mAb): anti-CD45—FITC/anti-CD14—PE, anti-CD3—FITC/anti-CD19—PE/anti-CD45—PerCP, anti-CD4—FITC/anti-CD8—PE/anti-CD3—PerCP, anti-CD3—FITC/anti-CD16+56-PE/anti-CD45—PerCP, and, as isotype control, γ1-FITC/γ2-PE/anti-CD45—PerCP. All antibodies were purchased from BD Biosciences, San Diego, CA, USA. Samples were incubated for 20 min at room temperature in the dark. After incubation, erythrocytes were lysed by suspending the cells in 500 μL of FACS lysing solution (BD) for 10 min. Cells were then washed with PBA (1% bovine serum albumin in PBS) and fixed using 1% formalin in PBS. To identify memory B-cell populations, peripheral mononuclear cells (PMBCs) were isolated using density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, St Louis, MO, USA). Isolated PBMCs were then stained with a mixture of anti-CD19—APC (BD) and anti-CD27—PE (BD) antibodies; samples were incubated for 20 min at room temperature in the dark. Next, the samples were washed using PBA and fixed in PBS containing 1% formalin.

Determination of CD154, ICOS, CD40 and TACI expression

CD154 and ICOS expression were assessed in activated T-cells. For CD154, PBMCs were cultured at 37 °C in a 5% CO2 environment in the presence of 10 ng/mL phorbol 12-myristrate 13-acetate (PMA) (Sigma-Aldrich, St. Louis Missouri, USA) and 1 μg/mL of ionomycin (Sigma-Aldrich) for 12 h at a density of 2×10^6 cells/mL in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MO, USA) supplemented with 10% foetal calf serum (PAA, Morningside, QLD, AU), 1 mM L-glutamine, 100 units/mL penicillin and 10 μg/mL streptomycin (GIBCO). After activation, PBMCs were stained with a mixture of anti-CD3—PerCP (BD), anti-CD154—PE (BD) and anti-CD69—FITC (BD). To examine ICOS expression, PBMC activation was performed overnight using the conditions described above. Cells were then stained with a mixture of anti-CD3—PerCP (BD), anti-ICOS-PE (BD) and anti-CD69—FITC (BD) following the protocol described above. Non-activated cells were used as negative controls for both CD154 and ICOS expression. CD69 expression was used as a positive control for T-cell activation. CD40 and TACI expression in B-cells were determined by staining PBMCs with a mixture of anti-CD40—PE (BD) or anti-TACI-PE (BD) and anti-CD19—APC (BD). Samples were acquired using a FACSCalibur® flow cytometer (BD); data analysis was

Table 1 Clinical and phenotypic characteristics of CVID patients.

Patient no.	Sex	Age (years)			Pneumonia episodes	Otitis episodes	Sinusitis episodes	Meningitis episodes
		Current	At onset	At Dx				
1	F	7	0.3	1.4	3	4	1	1
2	F	7	1.1	6.8	1	7	0	2
3	F	8	0.5	0.8	2	0	1	2
4	M	10	5	10.2	3	60	3	2
5	M	10	7	7.2	3	0	0	2
6	F	12	1	11.3	3	120	5	2
7	M	13	0.3	5.9	0	0	7	1
8	M	14	10.3	12.7	0	0	0	1
9	F	15	3	4	13	4	15	2
10	F	17	10	12.9	1	0	17	2
11	M	23	10	19	6	1	11	2
12	F	30	21	23	5	2	14	2
13	F	30	10	17	4	0	13	2
14	F	36	18	30	8	0	11	2
15	F	45	31	41	1	0	2	2
16	M	70	18	30	1	0	0	2

Gastroenteritis episodes	Allergic diseases	Bronchiectasis	Autoinmune diseases	Chronic diarrhoea	Splenomegaly	Chronic nonmalignat lymphoproliferation	Lymphadenopathy	Hepatomegaly
4	No	No	No	Yes	No	No	Yes	No
3	No	Yes	No	No	No	No	No	No
1	Yes	No	No	Yes	No	No	No	No
0	No	Yes	No	No	No	No	Yes	No
0	Yes	No	No	No	No	No	Yes	No
12	Yes	Yes	No	Yes	No	No	No	No
2	Yes	No	No	No	No	No	No	No
0	No	No	No	No	No	No	No	No
1	No	Yes	No	No	No	No	No	No
6	No	Yes	No	Yes	No	No	Yes	No
6	Yes	Yes	No	Yes	No	No	Yes	No
2	No	Yes	Yes	Yes	Yes	No	Yes	Yes
9	No	No	No	Yes	No	No	No	No
4	No	Yes	No	Yes	No	Yes	No	No
2	Yes	Yes	No	Yes	No	Yes	No	No
0	No	No	Yes	No	Yes	Yes	Yes	Yes

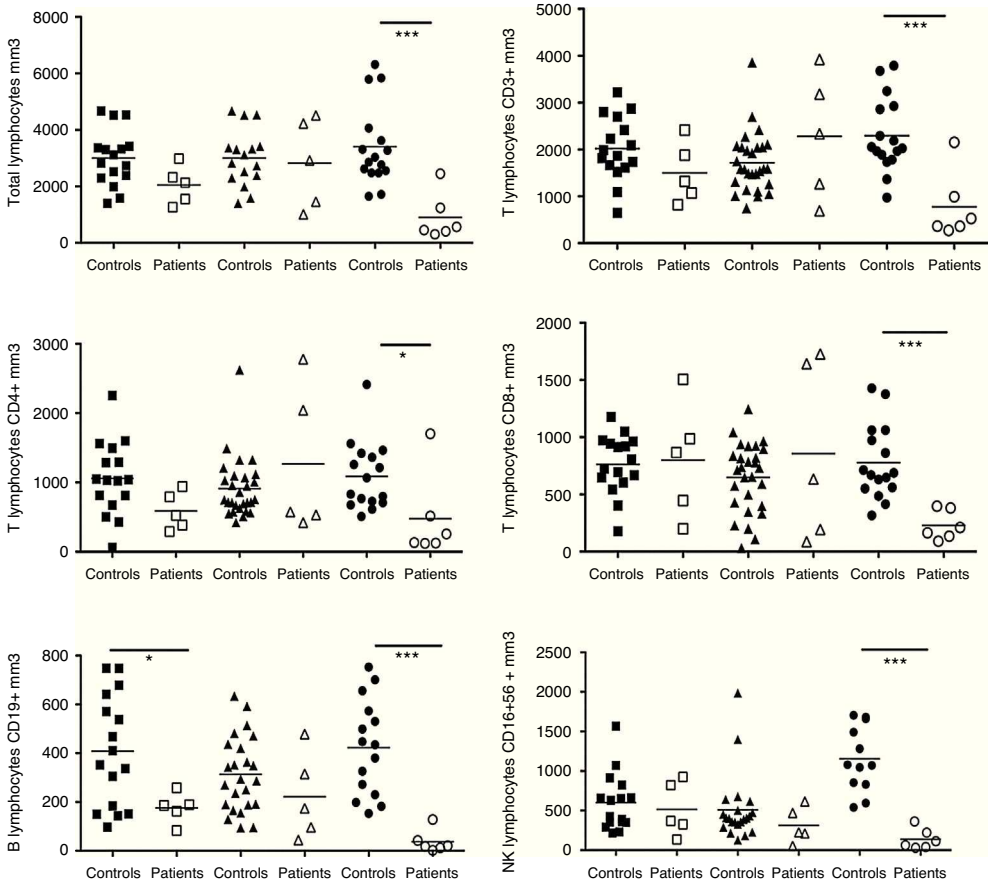

Figure 1 Analysis of lymphocyte populations. Flow cytometric analysis of total lymphocytes, T-cells, T cell subsets, B-cells and NK cells in absolute numbers (cells/mm^3) from children (□, $n = 5$), teenagers (△, $n = 5$) and adults (◯, $n = 6$) with CVID; these groups were compared with control children (■, $n = 17$); teenagers (▲, $n = 28$) and adults (●, $n = 16$). Differences between patients and controls were compared using the Mann–Whitney *U*-test. (*) Significant, $p < 0.05$; (**) very significant, $p < 0.01$; and (***) highly significant, $p < 0.001$.

performed using FlowJo 7.2.4 software (Tree Star. Inc., Ashland, OR, USA).

Statistical analysis

Descriptive data are presented as the mean ± standard deviation. We compared continuous variables between the age-matched groups using the Mann–Whitney *U*-test and nominal variables using Fisher's exact test. Differences between groups were considered significant when $p < 0.05$. Data were analysed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com)

Results

Patient population and clinical phenotype

Of the 16 CVID patients assessed, ten (63%) were female. There was one pair of siblings with CVID, and one patient had a relative with selective IgA deficiency; the demographic and clinical characteristics of the patients are summarised in Table 1. While the mean age of disease onset was 12.2 years, the mean age of diagnosis was 14.6 years. In CVID patents, the mean time from disease onset to diagnosis was 5.4 years (DS 4.2 years). Frequently observed clinical manifestations included pneumonia (88%, $n = 14$), sinusitis (75%, $n = 12$), chronic diarrhoea (75%, $n = 12$), bronchiectasis (56%, $n = 9$) and lymphadenopathy (37%, $n = 6$).

The immunological features of CVID patients are shown in Table 2, the median values of serum IgG, IgM and IgA at diagnosis were 286 mg/dL, 42 mg/dL and 18 mg/dL, respectively. The main feature that distinguished CVID patients was a reduced CD4/CD8 ratio (50%); however, two CVID patients (12.5%) had an exceptionally high CD4/CD8 ratio.

Age-related differences in lymphocyte levels in CVID patients

The absolute numbers of B, CD4, CD8, total T and NK cells from the patients were analysed and compared with the controls. The patients were divided into the following three groups based on age: children, 5–10 years old ($n = 5$); teenagers, 11–17 years old ($n = 5$); and adults, 17 years old and older ($n = 6$). These groups were compared with the following age-matched control groups: children

Table 2 Immunological features of CVID patients.

Patient no.	Age (years)	Sex	IgG (mg/dl)	IgM (mg/dl)	IgA (mg/dl)	Total leucocytes (mm^3)	Total lymphocytes (mm^3)	T lymphocytes (CD3+) (mm^3)	CD4+ lymphocytes (mm^3)	CD8+ lymphocytes (mm^3)	B lymphocytes (mm^3)
1	7	F	331	31	8	8400	1260	819	524	199	258
2	7	F	133	<17	<22	5500	2321	1880	940	865	162
3	8	F	495	49	13	5000	1550	1070	385	446	186
4	10	M	418	187	<25	7300	2131	1322	291	986	83
5	10	M	517	95	39	7100	2982	2415	792	1505	189
6	12	F	206	<16	<22	8900	4512	3925	2041	1727	315
7	13	M	476	51	<2	9000	4230	3185	2777	86	478
8	14	M	295	21	<25	4800	1008	689	420	193	174
9	15	F	293	16	4	8500	2919	2335	574	1642	44
10	17	F	261	<5	<6	11.200	1456	1267	532	634	96
11	23	M	154	<17	<25	4000	452	362	257	90	42
12	30	F	293	69	<25	14.400	2448	2154	1702	164	12
13	30	F	261	<17	<25	4600	566	526	131	381	18
14	36	F	344	<17	<23	4000	1240	992	516	397	128
15	45	F	43	19	7	3000	303	274	121	135	2
16	70	M	60	42	13	2400	408	363	124	210	20

NK lymphocytes (mm^3)	Monocytes mm^3	Ratio CD4/CD8 rv[1] 0.9–1.9	CD19+CD27+ (%)	CD19+CD27+ (mm^3)	CD19+CD27− (%)	CD19+CD27− (mm^3)	CD40/CD19 (%)	ICOS activated (%)	CD154 activated (%)	TACI (%)
135	109	2.6	8	21	82	212	85	44	96	84
369	149	1.1	48	78	52	84	88	10	69	48
326	105	0.86	30	56	70	130	88	38	ND	37
821	774	0.3	4	3	96	80	78	11	6	24
924	192	0.52	30	57	70	132	91	13	42	33
225	551	1.2	7	22	93	294	73	83	82	21
469	603	32.3	42	201	58	277	88	74	65	25
53	250	2.2	38	66	62	108	67	30	12	48
613	287	0.35	36	16	64	28	85	44	96	84
210	448	0.84	27	26	73	70	92	17	90	47
113	320	2.8	6	3	94	39	95	32	10	64
362	864	10.4	23	3	77	9	90	16	16	27
29	147	0.34	12	2	88	16	64	41	13	23
220	520	1.3	12	15	88	113	73	16	12	14
62	195	0.89	ND	ND	ND	ND	ND	16	96	ND
39	48	0.59	31	6	69	14	99	17	16	40

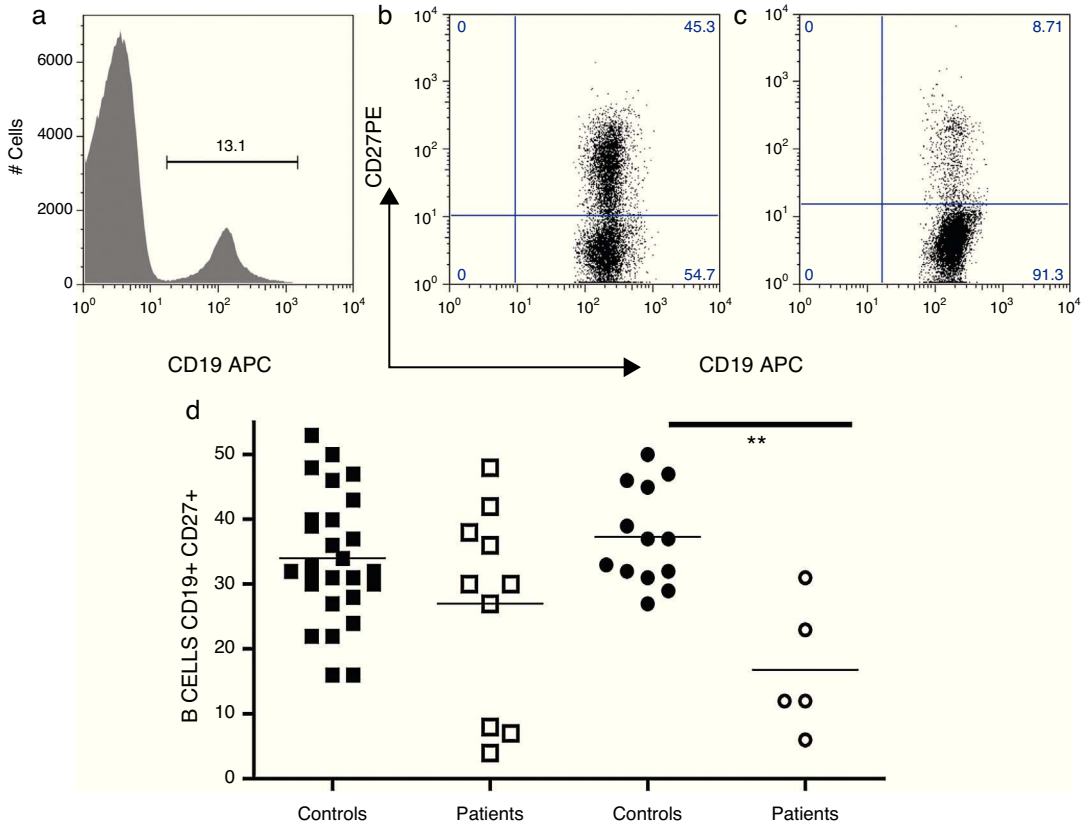

Figure 2 Analysis of B-cell subpopulations. Representative flow cytometric analysis of B-cell subsets. PMBCs were stained with mAbs against CD19 and CD27. After gating on lymphocytes according to forward (FSC) and side scatter (SSC), B-cells were identified according to CD19 expression (a). A CD19/CD27 dot plots were utilised to determine the frequency of the following B-cell subsets: total memory B-cells (CD19+CD27+) and naïve B-cells (CD19+CD27−). Controls (b) and CVID patients (c). Frequencies of total B memory cells from children and teenagers (◯, $n = 10$) and adults (□, $n = 5$) with CVID were compared with control children and teenagers (■, $n = 27$) and adults (●, $n = 13$). Differences between patients and controls were compared using the Mann–Whitney *U*-test (Fig. 2d). (*) Significant, $p < 0.05$; (**) very significant, $p < 0.01$; and (***) highly significant, $p < 0.001$.

($n = 17$), teenagers ($n = 28$) and adults ($n = 19$) (Fig. 1). Adult CVID patients exhibited a pronounced reduction in all lymphocyte populations compared with HD, including CD3 cells ($p = 0.0006$), CD4 T-cells ($p = 0.0245$), CD8 T-cells ($p = 0.0007$), CD19 B-cells ($p = 0.0002$) and CD16+56 NK cells ($p = 0.0001$). Children with CVID showed a moderate reduction in CD19 cells ($p = 0.0386$).

B-cell subsets in CVID patients

The frequency of memory B-cells in 15 CVID patients were examined; an adult patient was not examined because she had less than one percent of peripheral B-cells (Fig. 2). The patients were divided into two groups, children and teenagers ($n = 10$) and adults ($n = 5$), and compared with age-matched control children and teenagers ($n = 27$) and adults ($n = 13$). CD27 expression has been used as a surrogate marker for memory B-cells. Thus, as shown in Fig. 2a, the simultaneous evaluation of CD19 and CD27 enabled the identification of naïve (CD19+, CD27−) and memory B-cells (CD19, CD27+) in the peripheral blood. As shown in Fig. 2b, adult CVID patients showed significantly reduced levels of memory B-cells compared with age-matched controls ($p = 0.0036$).

CVID patients were also divided into two groups according to the frequency of memory cells. Group I comprised of patients whose memory B-cell frequencies were at least one standard deviation below the values obtained for memory B-cells from age-matched controls (mean B cells CD19+CD27+ of children and teenager controls=34%; DS = 9.8 and adult controls = 37%; DS = 7.5). Patients in Group II had normal or elevated numbers of memory B-cells. Table 3 shows a summary of the clinical manifestations of CVID in each group. Patients with a low frequency of memory B-cells showed a significant increase in the incidence of chronic diarrhoea ($p = 0.041$) and pneumonia ($p = 0.026$) compared with patients with normal or elevated frequencies of memory B-cells.

Analysis of CD40, CD154, ICOS and TACI expression in CVID patients

The frequency of co-stimulatory molecules expression is shown in Fig. 3. When B-cell CD40 expression was analysed, we found significant differences between 15 patients and

Table 3 Clinical manifestations of CVID patients grouped according to percentage of memory B cells.

Clinical characteristics	Group I ($n=7$)	Group II ($n=8$)	p value
Mean age	21	19	0.487
Mean age at onset of symptoms	9.3	6.3	0.684
Mean age at diagnosis	16	14	0.224
Number of patients with history of pneumonia	7	6	0.467
Mean event rate of pneumonia	**5**	**4**	**0.026***
Number of patients with complications of bronchiectasis	5	3	0.315
Number of patients with history of otitis	5	2	0.132
Mean event rate of otitis	37	6	0.152
Number of patients with history of sinusitis	7	4	0.077
Mean event rate of sinusitis	8	10	0.242
Number of patients with history of gastroenteritis infection	6	5	0.569
Mean event rate of gastroenteritis infection	6	3	0.061
Number of patients with chronic diarrhoea	**6**	**2**	**0.041***
Hospitalisations	7	8	1
Mean event rate of hospitalisations	6	4	0.101
Mean event rate of days of stay	59	63	0.955

*$p < 0.05$.

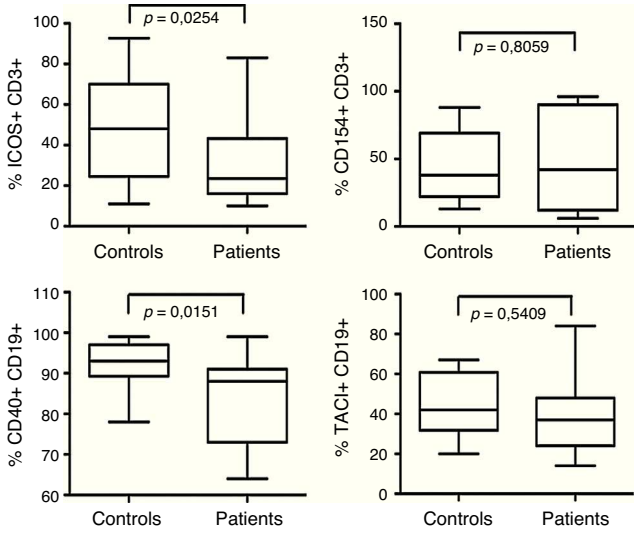

Figure 3 Analysis of CD40, CD154, TACI and ICOS expression. ICOS and CD154 expression was examined in activated CD3+ T-cells. ICOS expression was analysed from CVID patients ($n=16$) and controls ($n=17$). CD154 expression was analysed from CVID patients ($n=16$) and controls ($n=17$). TACI expression was analysed in CD19+ B-cells from CVID patients ($n=15$) and controls ($n=14$). B-cell CD40 expression was assessed in CVID patients ($n=15$) and controls ($n=18$). Dates are presented as box plots displaying medians, 25th and 75th percentiles and 10th and 90th percentiles as vertical lines. Differences between patients and controls were determined using the Mann–Whitney U-test.

18 controls ($p=<0.0151$). No differences were observed in CD154 expression in T-cells from 16 CVID patients compared with 17 controls ($p=0.8$). TACI expression was also similar between 15 CVID patients and 14 controls ($p=0.54$). However, ICOS expression on T-cells was reduced in the 16 CVID patients analysed compared with 17 controls ($p=0.0254$).

Discussion

CVID is a highly heterogeneous immunodeficiency syndrome in which B cell and T-cell defects have been shown.[17] In this study, eight out of 16 CVID patients showed significant alterations in CD4 and CD8 T-cell populations. Consistent with an observation by Wright,[18] these patients also exhibited reduced CD4/CD8 ratios.

A recent explanation for the decrease in CD4 T-cells in CVID patients involves spontaneous apoptosis and a reduction in thymus size. Because CVID is a progressive disease, patients who experience phenotypic changes such as reduction of T and B-cells can also develop complications over time.[17] Paediatric patients predominately showed a significant reduction in CD19 B-cells, whereas in adults, all lymphocyte populations were reduced. Although these differences between adult and paediatric patients have been described previously, no clear aetiopathogenesis has been established. The presence of both T-cell and B cell abnormalities suggests distinct disease mechanisms and indicates that different approaches for genetic research should be used. A molecular explanation for the differences observed in the absolute number of lymphocyte has not been described. However, it is possible that defects in several molecules may lead to flawed interactions between B and T-cells, potentially explaining the various clinical changes observed over time in CVID patients.[19] Importantly, a substantial defect was noted not only in the reduction of overall T cell population, but also in NK and B-cells; consequently, some of these patients could be considered as having combined immunodeficiency with a common early defect in lymphocyte differentiation.[20] Two patients with a form of adenosine deaminase deficiency have been reported to suffer from opportunistic infections in adulthood.[21] Therefore, this pathway should be further investigated in CVID patients.

Classification of B cell differentiation through the characterisation of circulating B-cell subpopulations is an important tool to understand the clinical manifestation in

CVID. Splenomegaly, granulomatous disease and infections have been associated with reduced levels of switched memory B-cells.[14,15] A limitation of this study was the lack of measurement of switched memory B-cells. However, we used CD27 expression as surrogate marker for memory B-cells. CD27 expression increases gradually with age; thus, in adults, approximately 40% of circulating B cells are CD27+.[5] However, we found that adult CVID patients had a significant reduction of memory B-cells compared with age-matched controls. This finding indirectly confirms previous results, indicating impaired germinal centre function over the progressive course of CVID.[22]

Among the CVID patients, we detected some with a significant reduction in the memory B-cell compartment (Fig. 2b). The patients with reduction in memory B-cells were classified in Group I. A relevant feature in these patients was the high prevalence of chronic diarrhoea and an increased number of episodes of pneumonia compared with CVID patients with normal levels of memory B-cells. Memory B-cells undergo somatic hypermutation and generate immunoglobulins rapidly and vigorously during a secondary immune response; their absence in some CVID patients may partially explain the higher prevalence of infections observed in CVID patients in Group I (Table 3).[23]

The clinical phenotype of CVID has been attributed to mutations in genes that encode molecules such as ICOS, TACI, CD19, BAFFR, CD21, CD20 and CD81. However, mutations in each of those genes are present in less than 20% of CVID patients.[24] All patients analysed in this study exhibited normal TACI expression; however, a functional evaluation and sequencing are needed to definitively rule out a genetic defect in *TNFRSF13B*.

Although ICOS expression on T-cells from CVID patients was detectable, it was significantly reduced compared with controls. Reduced ICOS expression may indicate an abnormal mechanism of communication between B and T-cells and play a key role in the pathogenesis of CVID.

In contrast with a previous report, the frequency of CD40 expression in B-cells from CVID patients was significantly reduced.[25] CVID patients not only had less B-cells, but B-cell CD40 surface expression was also diminished. Due to the important role of CD40 in B cell differentiation, this reduction in CD40 expression may account for some of the defects found in these patients.

In this study, we identified possible candidates for a more detailed analysis. For example, patients with low ICOS expression should be examined for mutations. Additionally, Btk should also be considered, as mutations in this enzyme have been reported to be responsible for a CVID-like disease that may represent a mild form of XLA.[26–28] In fact, one patient who was initially diagnosed with CVID had a mutation in Btk and was excluded from this study. Taken together, we believe that the insights gained in this work may help to improve the diagnosis of CVID. Further, similar to recently described mutations in CD20 and CD81 (7; 11), our findings may provide us with the opportunity to find new causes for CVID.

The diverse and widespread distribution of these defects further highlights the heterogeneity present among CVID patients and indicates that multiple factors likely play a role in generating the CVID phenotype.

Ethical disclosures

Confidentiality of data. The authors declare that they have followed the protocols of their work centre on the publication of patient data and that all the patients included in the study have received sufficient information and have given their informed consent in writing to participate in that study.

Right to privacy and informed consent. The authors have obtained the informed consent of the patients and/or subjects mentioned in the article. The research was conducted at National Institute of Pediatrics under approved protocol (#29/2009). The author for correspondence is in possession of this document.

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this investigation.

Conflict of interest

The authors have no conflict of interest to declare.

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