Journal of Autoimmunity xxx (2010) 1–8

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Autoimmunity in common variable immunodeficiency: Correlation with lymphocyte phenotype in the French DEFI study

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ARTICLE INFO

Article history:
Received 6 July 2010
Received in revised form
10 September 2010
Accepted 5 October 2010

Keywords:
Autoimmunity
Autoimmune cytopenia
Common variable immunodeficiency
B cells
T cells

ABSTRACT

Common variable immunodeficiency (CVID) is the most frequent clinically expressed primary immunodeficiency in adults and is characterized by primary defective immunoglobulin production. Besides recurrent infectious manifestations, up to 20% of CVID patients develop autoimmune complications. In this study, we took advantages of the French DEFI database to investigate possible correlations between peripheral lymphocyte subpopulations and autoimmune clinical expression in CVID adult patients. In order to analyse homogeneous populations of patients with precise clinical phenotypes, we first focused on patients with autoimmune cytopenia because they represent prototypic autoantibody mediated diseases. In a secondary analysis, we have tested our conclusions including all "autoimmune" CVID patients. We describe one of the largest European studies with 311 CVID patients, including 55 patients with autoimmune cytopenia and 61 patients with clinical or serologic autoimmune expression, excluding autoimmune cytopenia. We clarify previous reports and we confirm a very significant correlation between an increased proportion of $CD21^{low}$ B cells and CVID associated autoimmune cytopenia, but independently of the presence of other autoimmune disorders or of splenomegaly. Moreover, in CVID associated autoimmune cytopenia, T cells display an activated phenotype with an increase of HLA-DR and CD95 expression and a decrease in the naïve T cell numbers. Patients with other autoimmune manifestations do not harbour this "T and B cells phenotypic picture". In view of recent findings on $CD21^{low}$ B cells in CVID and RA, we suggest that both a restricted subset of B cells and a T cell help are required for a breakdown of B cell tolerance against membrane auto antigens in CVID.

1. Introduction

Common variable immunodeficiency (CVID) is the most frequent clinically expressed primary immunodeficiency in adults, and is characterized by low serum levels of IgG and usually of IgA or of IgM. As a consequence, patients can develop, often late in life, recurrent bacterial infections, mostly in the upper respiratory tract. This common clinical picture may result from multiple mechanisms. Although the vast majority of CVID patients do not have a defined genetic defect, failure of T cell/B cell-cooperation, primary T cell defects or primary B cell defects have been reported [1–17].

Beside infectious manifestations, other clinical complications in CVID patients have been largely described as chronic enteropathy, benign or malignant lymphoproliferation, granulomatous disease and autoimmune disorders including autoimmune cytopenia [18,19]. Thus, more than 20% of patients with common variable immune deficiency (CVID) have autoimmune complications which are poorly understood and in many cases difficult to manage on the clinical level [18,20–23]. The pathogenesis of autoimmunity in CVID has always been a paradox: autoantibodies or auto reactive B cells may be produced against some tissues whereas at the same time, few, if any, IgG are detected in the serum or after vaccinations. A way to address this paradox could be to identify in CVID patients lymphocyte sub-populations that could harbour auto reactive clones or reflect a pathway of abnormal activation of the immune system.

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doi:10.1016/j.jaut.2010.10.002

An European classification based on the main B cell abnormalities, which were the reduced percentages of B cells, of switched memory B cells (smB: $CD27^{+}IgD^{-}$) and an increase in the proportion of $CD21^{low}$ cells ($CD19^{hi}CD21^{-}CD38^{-}$) helped to differentiate patients with the diagnosis of CVID [24]. In a previous work, patients with reduced smB (<0.4% of lymphocytes) and increased $CD21^{low}$ (defined only on $CD19^{+}CD21^{-}$) B cells tended to have more autoimmune cytopenia and splenomegaly [[25], 40 CVID patients]; however, recently, the expansion of $CD21^{low}$ B cells pointed only patients with splenomegaly [[23], the EURO class study, 303 CVID patients]. T cell abnormalities in CVID associated autoimmune manifestations have also been suggested as a more pronounced decrease in regulatory T cell numbers [12,11] or a decrease in CD8T cell numbers [19]. The heterogeneity of the findings could be linked to the rarity of the pathology but one could also underline the difficulties in establishing homogeneous patient cohorts, considering the various potential manifestations of clinical or serologic autoimmunity.

In this study, we took advantage of the French DEFI database to investigate possible correlations between peripheral blood T and B cell subpopulations and autoimmune clinical expression in 311 adult patients with CVID [French DEFI study, [26]]. For each case, autoimmune manifestations have been recorded, and an extensive B and T lymphocytes immunophenotyping was centralized.

In order to analyse homogeneous populations of patients with precise clinical phenotypes, we first focused our analysis on patients with autoimmune cytopenia because they represent prototypic autoantibody mediated diseases and because they are the most frequent manifestations in CVID. In a secondary analysis, we have tested our conclusions including all "autoimmune" CVID patients.

The aims were to determine in the DEFI cohort clinical and immunologic phenotype of CVID patients with autoimmune manifestations 1) to test and possibly improve and unify previous data (see supra), 2) to test the hypothesis that common B and T cell abnormalities in different primary immunodeficiencies can be associated with autoimmunity.

2. Material and methods

2.1. Patients

DEFI is a French national study on adults with primary hypogammaglobulinemia.

Inclusion criteria are hypogammaglobulinemia with serum IgG level < 5 g/l, and/or IgA level < 0,7 g/l, and/or IgM level < 0,4 g/l, and/or IgG subclass deficiency. Ig levels are determined before any replacement therapy. Exclusion criteria are secondary hypogammaglobulinemia and refusal of consent for participation. The study has been approved by the local ethics committee and all patients gave written informed consent; the clinical database and serologic database are centralized in the Department of Clinical Immunology, Saint-Louis Hospital in Paris, France. A total of 313 patients with CVID were enrolled in this cohort between April 2004 and April 2008. For each patient, clinical file included the patient's main infectious, autoimmune, lymphoproliferative and tumoral complications. Autoimmune phenomena comprised: autoimmune cytopenia, pernicious anemia, thyroiditis, vitiligo, arthritis, systemic lupus, Sjögren syndrome, diabetes, polymyositis and "others". The systematic serologic screening for autoimmunity included direct Coomb's test, anti-nuclear antibodies (ANAs), anti-double stranded DNA antibodies (anti-dsDNA), cryoglobulinemia, and anti-phopholipids antibodies. From April 2004 through April 2008, 470 patients entered the study, and among them 313 were diagnosed with CVID based on the European Society for Immunodeficiency (ESID/PAGID) criteria (9). Considering the aim of our analysis, DEFI CVID patients were classified as: 1) NI group including patients without any clinical or serologic autoimmune manifestations. Patients with diabetes were excluded from all groups as the definition of the type of diabetes was not always available (2 patients) 2) Cy group, including patients with autoimmune cytopenia (peripheral thrombocytopenia with or without autoantibodies, autoimmune haemolytic anemia, and neutropenia) 3) AI group, including patients with any other manifestation (clinical or serologic) of clinical or serologic sign of autoimmunity than cytopenia.

Fifty healthy donors (HC group) were included for the phenotypic analysis: 25 males and 25 females with a median age of 36 years, and results were described previously (29).

2.2. Flow cytometric analysis

A blood sample collected on a single day at inclusion was used to determine complete B and T cell phenotypes at the same reference laboratory for all patients and controls (Immunology Laboratory, Assistance Publique Paris). In patients with substitutive immunoglobulin therapy, blood samples were collected just before Ig infusion. All analysis was performed within the next 24 hours. For immunofluorescence staining, fresh EDTA whole blood samples were stained at room temperature using predetermined saturating concentrations of antibodies (Abs) for 15 minutes and blood erythrocytes were lysed after staining using FACS Lysing solution (BD, CA, USA) according to the manufacturer recommendations.

The percentages and absolute values of the main lymphocytes populations were determined using the following antibodies CD45-FITC, CD3-FITC or -PerCP, CD4-PE or -APC Cy7, CD8-PerCP, -PE Cy7 or -APC, CD16/CD56-PE, and CD19-APC (all from BD Biosciences or Pharmingen, San Diego, CA, USA). For the B cell phenotypes, samples were washed in PBS1X − SVF 2% before staining. Antibodies used were CD19-APC (BD biosciences), IgD-FITC (Dako, Glostrup, Denmark), CD27-PE (BD biosciences), IgM-PC5 (BD Pharmingen), CD38-FITC (Immunotech, Marseille, France), CD21-PE (BD Pharmingen) and CD95-PE (BD Pharmingen). For the T cell phenotypes, percentages and absolute counts of competent ($CD28^{+}$), naive ($CD45RA^{+}CCR7^{+}$), and activated ($CD95^{+}$ or $HLA\text{-}DR^{+}$) T cells were analysed for CD4+ and CD8+ subsets; regulator T lymphocytes ($CD4^{+}CD25^{+}CD127^{low}$) were analysed as well. Antibodies used were CD45-FITC, CD3-FITC or -PerCP, CD4-PE or -APC Cy7, CD8-PerCP, -PE Cy7 or -APC, CD28-PE, HLA-DR-PE Cy7, CD38-APC, CD45RA-APC, CD95-FITC, CD127-PE, CD25-PE (BD Biosciences or Pharmingen) and CCR7-PE (RD, Minneapolis, USA).

For the analysis, the cells were first gated on the lymphocyte population based on forward and side scatter characteristics. The data acquisition was performed on FACSCanto™ analyser (BD Biosciences, San Diego, CA, USA) and 10^4 gated lymphocytes were analysed on Diva software (BD Biosciences).

2.3. Statistical analysis

Descriptive analysis used median range for clinical values and medians with interquartile range (IQR) values for all other values. The primary endpoint of the study was to compare characteristics of patients with CVID and autoimmune cytopenia (Cy group) to patients with CVID without any autoimmune manifestations (NI group), using nonparametric Wilcoxon rank-sum test for continuous variables, and a Pearson's chi-quare test or Fischer's exact test for frequencies. The Benjamini and Hochberg [29] procedure was used to correct for multiple comparisons, and p values < 0.023 were considered significant. A secondary analysis was performed, without adjustment for multiple test, to compare characteristic of patients with autoimmune cytopenia (Cy group) to patients with clinical or serologic autoimmune manifestation excluding cytopenia

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(AI group) and to patients without any autoimmune manifestations (NI group), using a non-parametric Kruskall–Wallis test, and a Pearsons's chi-square test or a Fischer's exact test, as appropriate. All reported p values are two sided. Statistical analysis was performed using Stata version 11 (Stata Statistical Software, Stat Corp., Texas, USA).

3. Results

3.1. Patient population

Among the 311 CVID patients registered, 116 (37%) presented clinical or serologic autoimmune manifestations (Cy and AI groups). 84 patients (27%) presented clinical manifestations and 32 (10,1%) only serologic expression of autoimmunity, as described in Table 1.

NI group included 195 patients (62.7%) with no clinical or serologic expression of autoimmunity. 112 patients were female and 83 male. The mean age at CVID diagnosis was 34 years (22–47). 25% patients developed splenomegaly and 13% granulomatous disease.

Cy group included 55 patients (17.7%) with autoimmune cytopenia. Among them 29 were male and 26 were female. Median age at CVID diagnosis was 29 years (16–46) and cytopenia revealed the immunodeficiency in about 75% patients (data not shown). 41 patients (74%) suffered from autoimmune thrombocytopenia (ITP), 17 patients (31%) suffered from autoimmune haemolytic anemia and direct Coomb's test was always positive, 10 patients (18%) suffered from neutropenia. 36 patients in this group developed splenomegaly (65%) and 8 patients developed a granulomatous disease (14%). Thus, as previously described [23], cytopenia in CVID is strongly associated with splenomegaly (see Fig. 1).

AI group included 61 patients (19.6%) with clinical or serologic autoimmune expression excluding autoimmune cytopenia.

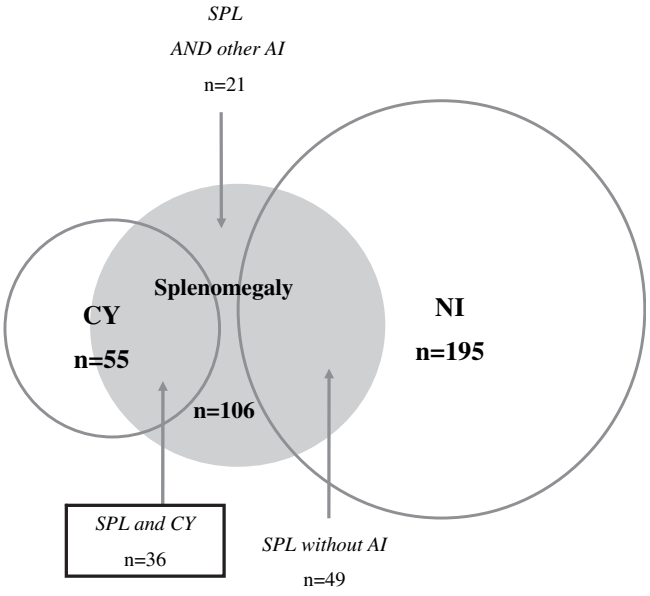

Fig. 1. Coincidence of splenomegaly with autoimmune cytopenia (Cy group) and other autoimmune manifestations (AI) group in patients with CVID. The diagram indicates the coincidence of splenomegaly in NI, Cy and AI groups.

The characteristics of these patients are summarized in Table 1. Mean age at CVID diagnosis was 43 years (30–58) with 39 female patients and 22 male. In this group, 21 patients developed splenomegaly (34%) and 8 developed granulomatous disease (13%).

3.2. Lymphocytes subsets and Ig production for Cy and NI patients

As shown in Table 2a, the absolute numbers of B cells and T cells were comparable in the NI and Cy groups, although decreased in all CVID patients when compared to healthy controls [28].

Immunoglobulin levels, especially IgG and IgA levels are reduced in patients with CVID (see Table 2a). However, despite absolute comparable numbers of B cells, we observed significantly higher IgG levels in Cy patients when compared to NI patients (median 3,1 g/l versus 1,9 g/l for NI patients, respectively; $p = 0.014$).

3.3. B cell subpopulations in CIVD with autoimmune cytopenia

Considering that autoimmune cytopenia are autoantibody mediated diseases, B cell activation in CVID could result either from the expansion of activated B cell subsets that are normally present in the peripheral blood of healthy individuals (such as pre-germinal center B cells) or from the abnormal activation of B cell subsets that are normally not activated (such as naïve or transitional B cells). In the circulating human B cell pool, it is classically possible to

Table 1
Clinical and serologic parameters of the 311 CVID patients (two diabetic patients were excluded).

	NI group	Cy Group	AI Group
Number of patients	195	55	61
CVID diagnosis (median age, years)	34 (22–47)	29 (16–46)	43 (30–58)
AI Symtptoms (median age, years)		26.48	
Sex ratio (F/M)	112/83	26/29	39/22
Haemolytic anemia	–	17	–
Thrombocytopenia	–	41	–
Neutropenia	–	10	–
Sjögren syndrom		2	11
Thyroiditis	–	1	11
Vitiligo	–	3	9
Rhumatoid arthritis	–	2	6
Systemic Lupus Erythematosus	–	1	0
Others		5	10
Direct Coomb's test	–	19	9
Antiphospholipid antibodies	–	5	15
Anti-nuclear antibodies	–	4	12
Anti-dsDNA Abs	–	1	2
Cryoglobulinemia	–	3	5
Biol. AI manifestation only	–	–	32
Splenomegaly	49	36	21
Granulomatous disease	25	8	8
Patients under Ig replacement	152	39	49

NI group: patients without clinical or biological autoimmune manifestations; Cy group: patients with autoimmune cytopenia, including peripherical thrombocytopenia, autoimmune haemolytic anemia, and neutropenia; AI group: patients with any manifestation of clinical or biological autoimmunity, excluding cytopenia. It should be noticed that in both Cy and AI groups, patients can display more than one clinical or serologic autoimmune manifestation (Table 1). For example, among AI patients, 3 patients develop Sjögren and thyroiditis.

Table 2a
Main lymphocyte populations and serum immunoglobulin levels for patients of NI and Cy groups.

	NI group, $n = 195$	Cy group, $n = 55$
Total B lymphocytes (10^6/l)	99 [45–176]	102 [20–221]
Total T lymphocytes (10^6/l)	1093 [810–1472]	1255 [903–1891]
IgG (g/l)	1.9 [0.72–3.71]	3.1 [1.59–3.97]*
IgA (g/l)	0.2 [0.07–0.34]	0.2 [0.04–0.36]
IgM (g/l)	0.2 [0.11–0.43]	0.27 [0.15–0.42]

Values are given as the median [interquartile range] for continuous variables.
*$p = 0.014$.

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Table 2b
B cell subpopulations for patients of NI and Cy Groups.

	NI group, $n = 195$	Cy group, $n = 55$
$CD19^+$ B cells (10^6/l)	99 [45–176]	102 [20–221]
$CD27^-$ IgD^+ Naïve Mature (% CD19)	79 [63–89.6]	84.5 [73.5–90.5]
IgM^{++} $CD38^{++}$ Transitional (% CD19)	1.5 [0.3–3.7]	1.1 [0.2–3]
$CD27^+$ IgD^+ Marginal zone (% CD19)	13 [6–24]	8.8 [3–16.5]
$CD27^+$ IgD^- Switched Memory (% CD19)	3 [1–7]	2 [0.7–4]
$CD27^-$ IgD^- Non CD27 Memory (% CD19)	2 [1–4]	2.80 [1.9–5.5]
$CD38^{++}IgM^-$ Plasmablasts (% CD19)	0.1 [0–0.3]	0 [0–0.2]
$CD19^{hi}CD21^-CD38^-$ (%CD19)	5 [2.2–12]	12 [6–21]*
$CD95^+$ (% CD19)	13 [6–26]	22 [9.7–33]

Values are given as the median [interquartile range] for continuous variables.
All values for B cell subpopulations are given as percentages in the CD19 gate.
*$p = 0.0002$.

distinguish naïve $CD27^-IgM^+IgD^+$ B cells, from $CD27^+IgM^+IgD^+$ marginal zone like B cells and $CD27^+IgD^-$ switched memory B cells, $CD38^{++}IgM^{++}$ transitional B cells from $CD38^{++}IgM^-$ class switched plasmablasts.

Table 2b summarizes B cells subpopulations data in the NI and the Cy patients groups:

As previously described in other studies, in DEFI CVID patients, the main abnormality of the B cell phenotype is a decrease of $CD27^+IgD^-$ switched memory B cells [28]. Warnatz et al. [24] correlated the frequency of $CD27^+IgD^-$ B cells in CVID and the *in vitro* IgG synthesis. Thus, one could have expected a higher number of these cells in autoantibody mediated diseases. However, we did not find any significant difference in the IgD^- $CD27^+$ percentages between Cy and NI patients. Recently, a new population of memory B cells lacking both expression of CD27 and IgD was associated with autoimmune phenomena [30]. In our study, we did not detect any variation in this population (Table 2b).

Neither $CD38^{++}IgM^{++}$ transitional B cell nor $CD38^{++}IgM^-$ class switched plasmablasts B cell compartments differ between the two groups. Considering classical parameters of B cell differentiation and activation, the almost significant difference between Cy and NI patients is a decrease in Cy patients$CD27^+IgM^+IgD^+$ marginal zone B cell proportion ($p = 0.025$).

3.4. $CD19^{hi}CD21^-CD38^-$ B cells are expanded in CVID autoimmune cytopenia compared to non-immune CVID patients

Warnatz et al. correlate on 40 CVID patients, the proportion of $CD21^-$ B cells with splenomegaly and autoimmune cytopenia. The $CD19^{hi}$ $CD21^{-/low}$ B cell population could be separated in two subsets [25]: one of chronically activated like B cells ($CD19^{hi}$ $CD21^{lo}IgM^{lo}CD24^{lo}CD38^{lo}$) and one of immature like B cells ($CD19^{hi}CD21^{lo}$ $IgM^{hi}CD24^{hi}CD38^{lo}$). At present, the precise function and origin of the $CD21^{low}$ cells in CVID remain obscure; nevertheless, today, the phenotype of this population is usually defined on $CD19^{hi}CD21^{-/low}CD38^{-/low}$ [31] They share the low surface expression of CD21 and CD23 with immature transitional circulating B cells [31,32] but in contrast to them, CD21low cells in CVID express low levels of CD38 [31]. Recently, it has been suggested in different studies [31,32] that they could be a completely unique B cell subpopulation. We confirm on a large serie that a $CD21^{low}$ ($CD19^{hi}CD21^-CD38^-$) B cell expansion correlates very significantly

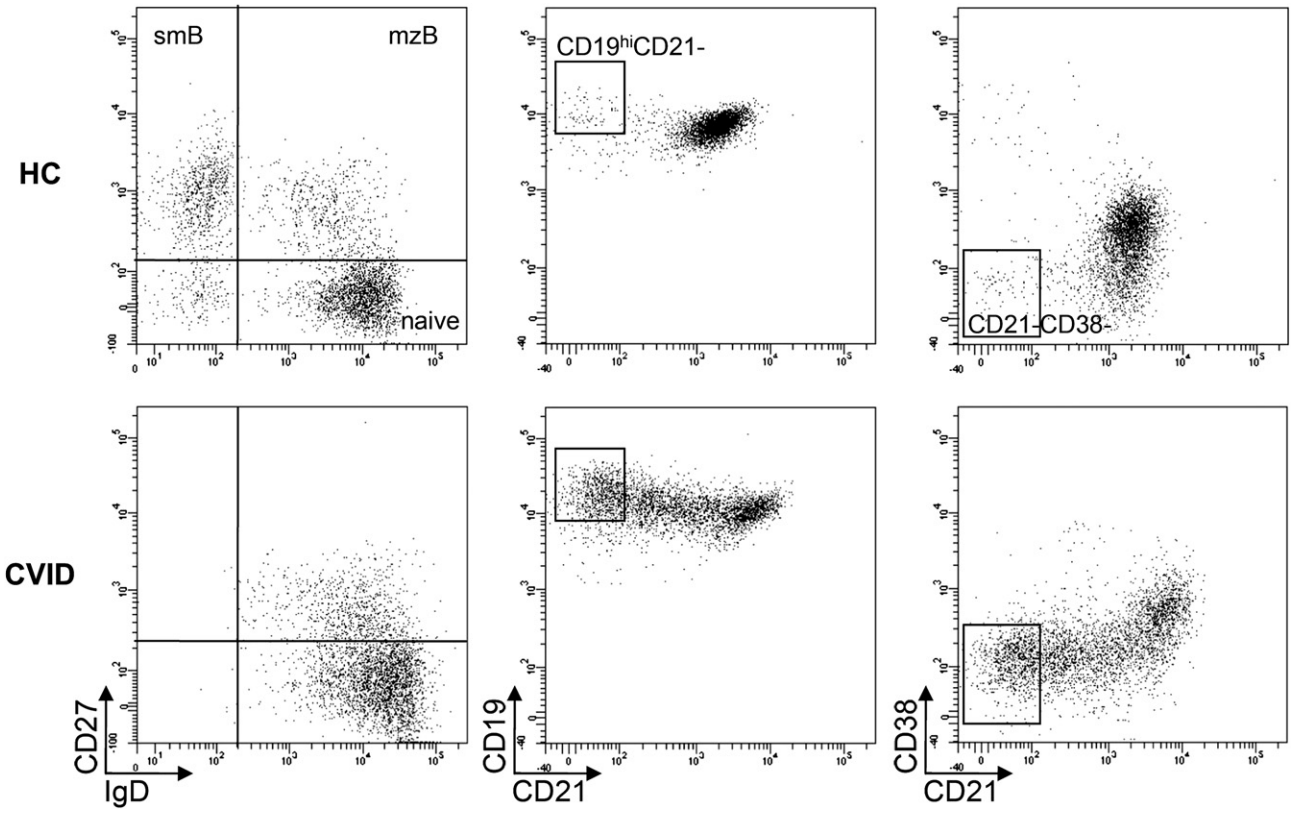

Fig. 2. Flow cytometry analyses of B cell subsets. The main B cell phenotypes in healthy controls (HC, top) and in CVID patients (bottom) are presented for one representative experiment. The B cell compartment was gated on CD19-APC positive cells. The distribution of naive ($CD27^-IgD^+$), mzB (marginal zone B-cells: $CD27^+IgD^+$) and smB (switched memory B: $CD27^+$ IgD^-) cells was evaluated using anti CD27-PE and anti IgD-FITC mAbs (left dot-plots). The $CD21^{low}$ population was evaluated by the intersection of the $CD19^{hi}CD21^-$ and $CD21^-CD38^-$ populations (middle and right dot-plots) using anti CD19-APC, anti CD38-FITC and anti CD21-PE mAbs. Flow cytometry was realized on a FACSCanto™ analyser using Diva software (BD biosciences).

Table 2c
T cell subpopulations for patients of NI and Cy Groups.

	NI group, $n = 195$	Cy group, $n = 55$
$CD4^+$ T cells (10^6/l)	565 [410–778]	650 [395–911]
$CD4^+$ CD45RA (% $CD4^+$)	29.6 [18.8–45.3]	16.2 [10.8–34.9]*
$CD4^+$ CD45RA CCR7+ (% $CD4^+$)	25.6 [12.4–42.5]	11.3 [4.5–28.7]**
$CD95^+CD4^+$ T cells (% $CD4^+$)	75.8 [59.9–89.6]	90.8 [78.5–96.3]***
$HLADR^+$ $CD4^+$ T cells (% $CD4^+$)	12.8 [8.3–24.4]	20.4 [11.5–27.3]
$CD8^+$ T cells (10^6/l)	458 [299–626]	520 [340–847]
$CD8^+$ $CD45RA^+$ (% $CD8^+$)	58.5 [46–69]	62.5 [48–75]
$CD8^+$ $CD45RA^+$ CCR7+ (% $CD8^+$)	18.8 [7.7–35.8]	16 [6–30]
$CD8^+$ $CD95^+$ (% $CD8^+$)	78.1[62–91]	78 [63–90]

All values for T cell subpopulations are given as percentages in the CD4 or CD8+ T cell gate.
Values are given as the median [interquartile range] for continuous variables $^*p = 0.001$, $^{**}p = 0.0005$, $^{***}p = 0.0001$.
NI group: patients without clinical or biological autoimmune manifestations; Cy group : patients with autoimmune cytopenia, including peripherical thrombocytopenia, autoimmune haemolytic anemia, and neutropenia.

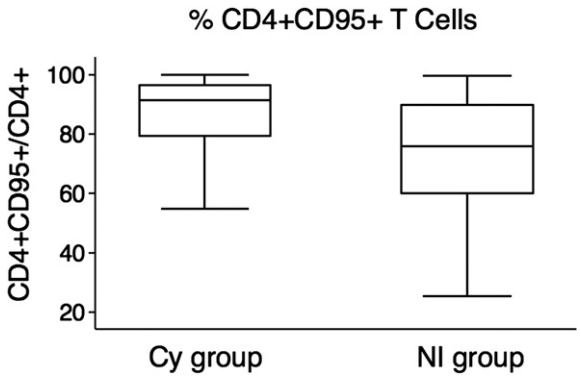

Fig. 3. Flow cytometry analysis of CD95+CD4+ T cell subsets from patients of Cy and NI groups. The T cell compartement was gated on CD3-PerCP positive cells. The distribution of $CD95^+$ $CD4^+$ T cells was evaluated using CD4-PE and CD95-FITC mAbs. Data are represented as box plots displaying medians, 25th and 75th percentiles as boxes (percentages of $CD95^+$ cells in the $CD4^+$ T cell gate), and 10th and 90th percentiles as whiskers. $p < 0{,}001$.

with autoimmune cytopenia ($p = 0{,}0002$, Fig. 2 and Table 2b). Moreover, among Cy CVID patients, we did not find any significant difference between patients with or without splenomegaly in term of $CD19^{hi}$ $CD21^-CD38^-$ B cells (Cy patients without splenomegaly: 11.8% [5–16.8]).

3.5. T lymphocyte phenotype

Several phenotypic and functional parameters of T cell-mediated immunity have already been studied in previous series of CVID [11–13,16,17,23]. In fact, a number of T cell defects have been demonstrated in a still undefined proportion of patients, including a decrease in T cell numbers especially for naïve T cells, a disruption of $CD4^+$ and $CD8^+$ TCR repertoire, an altered cytokine production and in some cases an increase of T cell turnover with expression of activation and apoptosis markers. In DEFI CVID patients, T cell numbers are indeed significantly lower with a significant decrease in naïve $CD4^+$ T cells [28]. Table 2c details the different subsets of T cells in Cy patients compared to NI patients. No statistical difference was found between absolute CD4T cell numbers in Cy group compared to NI group (median, 649×10^6/L versus 565×10^6/L, respectively; $p = 0{,}19$). However, we observed a significant decrease in the Cy patients naïve T cell proportions ($p = 0.005$). The numbers of regulatory T cells ($CD4^+$ $CD25^+$ $CD127^{low}$ T cells) seems to decrease in the Cy patients group, but this phenotype was available only for 17 patients out of the Cy group (6,8% [5.1–8]) and 65 patients out of the NI group (3,9% [2,4–6.1]).

3.6. Autoimmune cytopenia in CVID is significantly associated with an increase of CD95 expression on CD4+ T cells

FAS (CD95) belongs to the subgroup of the tumour necrosis factor receptor (TNF-R) family that contains a death domain and triggers apoptosis. However, Fas signalling pathway has also been implicated in non-apoptotic processes, including cellular activation, differentiation and proliferation, particularly for T and B lymphocytes. Thus, CD95 expression has been sporadically studied both in immune deficiencies and autoimmune diseases. An increase in CD95 expression on T or B cells has been described in some cases of CVID [13] and recently CD95 identified a subset of $CD27^-IgD^-$ memory B cells in SLE [33]. In comparison to controls, globally, CVID patients presented a higher frequency of $CD95^+$ T cells for both $CD4^+$ and $CD8^+$ T cells and for B cells [not shown, [28]]. Considering Cy CVID patients, the percentage of $CD4^+$ T cells expressing the CD95 marker increased compared to NI patients (91% in Cy group versus 75,7% in NI group $p = 0{,}001$, Table 2c and Fig. 3). This increase does not appear to be intrinsic to all $CD3^+$ cells as $CD8^+$ $CD95^+$ cells percentages were comparable in Cy and NI patients group (79,4% in the Cy group, versus 78,1% in the NI group, Table 2c). In addition, HLA DR expression appears also to increase on $CD4^+$ T cells suggesting that the increase of $CD95^+$ and HLA-DR^+ on $CD4^+$ T cells is a characteristic of Cy patients compared to NI CVID patients. Considering CD95 expression on B cells, it is noteworthy that $CD95^+$ B cell frequency slightly increases in the Cy patients group, compared to NI patients (Table 2b).

3.7. B and T cell abnormalities in CVID autoimmune cytopenia are rather specific and do not concern other autoimmune manifestations

IV Ig have been suggested to modulate Fas but not DR expression on CD4+ T cells [34]. Although the proportions of treated patients in each group (NI, Cy, AI groups) were rather equivalent (Table 1), we have checked the more significant parameters ($CD21^{low}$ B cells, $CD4^+CD95^+$ cells) for patients with autoimmune cytopenia that were on Ig replacement and for patients that were not. This analysis confirms our data independently of Ig replacement ($CD21^{low}$ B cells: non-substituted patients 11% [9–16.6] versus substituted patients 13% [5–24]; $CD4^+CD95^+$ cells: non-substituted patients 88,5% [76.8–93.1] versus substituted patients 92.2% [79–97]).

Finally, one of the most interesting findings in this study came from the comparison between Cy patients, NI patients and AI patients. AI patients include patients with any serologic or clinical autoimmune manifestation except autoimmune cytopenia. As shown in Table 3, the absolute numbers of B and T cells were comparable in NI, Cy and AI groups. Patients with autoimmune manifestations (Cy and AI groups) display significantly higher levels of IgG, compared to CVID NI patients ($p = 0.006$). However, this comparison clearly individualizes a "T and B cells phenotypic picture" of patients with autoimmune cytopenia including expanded $CD21^{low}$ B cells and decreased naïve T cells, associated with a peculiar increase in CD95 expression, especially on CD4+ T cells. It should be underlined that these parameters are very similar in NI CVID patients and AI patients.

4. Discussion

The current study aimed to investigate the composition of some B and T cell subsets in patients with CVID and autoimmune

Table 3
Comparison of IgG and main lymphocyte subpopulations of interest in the three groups.

	NI group, $n = 195$	Cy group, $n = 55$	AI group, $n = 61$	p
IgG (g/L)	1,9 [0,72–3,71]	3,1 [1,59–3,97]	3,2 [1,6–4,1]	0.006*
$CD19^+$ B cells (10^6/l)	99 [45–176]	102 [20–221]	88 [46–164]	0.92
$CD27^+$ IgD $^+$ Marginal Zone B cells	13 [6–24]	8.8 [3–16,55]	13 [6–24]	0.086
$CD19^{hi}$ $CD21^-$ $CD38^-$	5 [2.2–12]	12 [5–21]	5 [2–12]	<0.001*
$CD95^+$ B cells	13 [6–26]	22 [9,7–33]	14 [7.2–24]	0.058
$CD4^+$ T Cells (10^6/l)	565 [410–778]	650 [395–911]	544 [379–779]	0.362
$CD4^+CD45RA^+$	29.6 [18,8–45.3]	16.2 [10.8–34.9]	31.4 [20.1–43.1]	0.002*
$CD4^+CD45RA^+$ CCR7+ Naive $CD4^+$ T Cells	25.6 [12.4–42.5]	11.3 [4.5–28.7]	29.3 [19.3–39.9]	0.001*
$CD95^+CD4^+$ T Cells	75.7 [59.9–89.6]	91 [79–96]	72.5 [59.7–83.7]	<0.001*

Values are given as the median [interquartile range] for continuous variables. Asteriks indicate a significant difference ($p < 0.025$) between one and others of the three groups. Difference between the groups were compared by the Kruskall–Wallis test.
NI group: patients without clinical or biological autoimmune manifestations; Cy group : patients with autoimmune cytopenia, including peripherical thrombocytopenia, autoimmune haemolytic anemia, and neutropenia; AI group: patients with any manifestation of clinical or biological autoimmunity, excluding cytopenia.

cytopenia. We expected that it could help to understand the mechanisms of autoimmunity during CVID and more generally to approach the pathogenesis of some autoimmune diseases.

For this purpose, we have used data from 311 CVID patients from the French DEFI study to get into significant conclusions. 37% of the patients with CVID in DEFI study have autoimmune complications and 17.6% have autoimmune cytopenia. In the previous clinical series of CVID subjects, the percentages of autoimmune complications were comparable although varying between 20 and 40% [18–20,23,24]. Many forms of autoimmunity were in fact considered, including seropositive or negative arthritis, SLE, organ-specific diseases, cytopenia and diverse serologic manifestations. We have chosen to focus on autoimmune cytopenia in our analysis as CVID frequently associated "prototypic" autoantibodies mediated diseases with an easy non-overlapping clinical and serologic definition. In the previous series of CVID patients, the percentages of autoimmune cytopenia were also comparable to our analysis, although slightly inferior [about 10%, 18–20]. From our study and others, it is obvious that cytopenia is associated with an increased frequency of splenomegaly with an unclear physiopathological link.

We first showed that in the Cy group, there is no difference in the total numbers of B and T lymphocytes, but there is a significantly higher level of serum IgG compared to the NI group. To complete our study, we isolated an AI group, including all CVID patients with clinical or serologic autoimmune situations, excluding autoimmune cytopenia. This increase was also noticed in the AI group. The significance of these higher serum IgG levels during autoimmune conditions associated with CVID remains to be determined. It may be directly linked to the mechanism of the autoimmune disease, but may rather be explained by the early occurrence of autoimmune cytopenia in the course of CVID.

Several groups have reported abnormalities in peripheral blood B cell homeostasis in patients with CVID and autoimmune expression including autoimmune cytopenia or splenomegaly. Flow cytometry analysis identified the expansion of an unusual B cell subset in CVID patients, characterized by a very low expression of CD21 (CR2, complement receptor 2). M. Rakhmanoz et al. [31], and I. Isnardi et al. [32] reported an extended phenotype for these cells, distinct from other circulating B cells. These B cells express a polyclonal and unmutated BCR repertoire and, although not normally reactive in vitro, are able to produce higher levels of IgM than naïve B cells under certain conditions of stimulation. In the very recent study of I. Isnardi et al. [32], the $CD21^{low}$ B cell subset from healthy donors, and from some RA and CVID patients appears to be enriched in polyreactive and in a few more auto reactive clones, expressing autoantibodies that recognize mostly cytoplasmic structures. We described a very significant association between $CD21^{low}$ B cells and CVID associated autoimmune cytopenia, but independently of the presence of a splenomegaly. It should be underlined that in the AI group, the percentage of $CD21^{low}$ B cells is very similar to the percentage observed in the NI group, suggesting a specific link between autoimmune cytopenia physiopathology in CVID and some characteristics of the $CD21^{low}$ B cell subset. This subset being present in normal individuals, one could ask the question of its responsibility in the production of pathogenic anti-platelets, anti-RBC or anti-neutrophils during non immunodeficiency associated autoimmune cytopenia. However, in such diseases, pathogenic antibodies are classically isotype switched and harbour somatic mutations. Moreover various studies suggest that the development of platelet-reactive or Rh-autoantibodies is driven through clonal expansion of B cells using genetically restricted heavy and light chain products [35–38]. Thus, in these settings, it would be of great interest to explore BCRs characteristics of $CD21^{low}$ B cells in patients affected with CVID and autoimmune cytopenia.

Various stimuli are susceptible to influence the silencing of developing auto reactive B cells, including T cell help, TLRs activation and other direct survival/apoptotic signals [39]. In this study, we showed a very significant increase of $CD95^+$ and HLA-DR^+ $CD4^+$T cells in CVID patients with autoimmune cytopenia compared to NI CVID patients. These T cell abnormalities take place in a context of more pronounced naïve T cell depletion. We would like to suggest that in CVID patients with autoimmune cytopenia, T cells could provide a B cell help at different levels.

First, CVID patients, as well as patients with lymphopenia, may display a more "auto reactive" T cell repertoire than healthy individuals. Peripheral T cell lymphopenia is known to increase the capacity of T cells to expand with auto reactive T cells and also to reduce the efficiency of deletion mechanisms in response to self-antigens [40–42]. The decrease in naïve CD4+ T cells in Cy patients compared to NI patients is likely due to a T cell maturation process since total numbers of CD4+ T cells remain constant in both groups. Second, Fas is a death receptor and both B cells and T cells in CVID patients have been described to display a higher level of CD95 expression compared to healthy controls [14,16,17]. Whether cause or consequence of the primary immunodeficiency, the increase of Fas expression in CVID appears to be associated with an enhanced apoptosis and a decreased survival of both T and B cells [14,16,17,32]. The increased susceptibility to apoptosis and enhanced CD95 expression on CD4+ T cells have been suggested to be events that could contribute to several diseases like systemic lupus erythematosus (SLE) [43–45] and HIV infection. Although the molecular mechanisms underlying these processes remain obscure, we can underline that both SLE patients and HIV patients display an abnormal B cell activation. The increased Fas expression and HLA-DR expression on $CD4^+$ T cells in CVID patients with autoimmune cytopenia might reflect a persistent activated status which could be

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linked to a T-B cell cooperation [for review see [44]]. In this setting, it is interesting to note that Giovanetti et al. described a positive correlation between the percentage of CD4+ apoptotic cells and Ki-67$^+$ CD4$^+$ cells, with a negative correlation with percentage of naïve T cells for some CVID patients [[13], this study included 60 patients]. Third, the number and frequency of regulatory T cells have been recently investigated in different subgroups of CVID clinical manifestations: patients with autoimmunity or lymphoid proliferation showed a significant reduction in the frequency and the numbers of regulatory T cells [12,27 CVID patients, 10 patients with autoimmune diseases]. We did not find such difference in our work, focusing only on CVID patients with autoimmune cytopenia (17 patients), but this point will need to be clarified on a larger number of patients.

As a conclusion, autoimmune cytopenia appears to be the most frequent expression of autoimmunity in primary immunodeficiency, independently of their mechanisms. Subjects with X-linked hyper-IgM and a defect in isotype switching and somatic mutation process, have an increased number of self-reactive clones expressing auto reactive BCRs, and are likely to develop autoimmune thrombocytopenia [46,47]. CVID patients develop autoimmune cytopenia and harbour various genetic defects in B or T cell differentiation or activation pathways. Thus, removal of B cells reactive against membranous blood cell antigens, could require not only sufficient and normal B cell receptor signalling, but also additional cell mediated events. In this view, our analysis is the first to clearly differentiate autoimmune cytopenia from other autoimmune manifestations in CVID in terms of B and T cell phenotype. Our results suggest that both a restricted subset of B cells (CD21$^{low/-}$ B cells) and a T cell help (presence of activated T cells, maybe associated with a restricted T cell repertoire and a loss of regulatory T cells) could be required. Of course, further investigations, especially at the clonal level, seem necessary to complete these data.

Acknowledgments

This study was supported by the National Program for Clinical Research, PHRC 2005 and by the National Center on Hereditary Immune Deficiencies, CEREDIH.

We thank Honey Levallois and R Alles for critical reading of the manuscript.

Appendix

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