Influence of Splenomegaly and Splenectomy on the Immune-Cell Profile of Patients with Common Variable Immunodeficiency Disease

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Abstract

Purpose About 25% of patients with common variable immunodeficiency disease (CVID) have splenomegaly necessitating splenectomy but its consequences on the immunological profile of CVID patients have never been studied. We analyzed 11 CVID patients' comprehensive blood immune-cell phenotypes before and after splenectomy.

Methods Flow-cytometry analyses of immune-cell populations.

Results Among 89 CVID-cohort patients, 41 with splenomegaly, splenomegaly was strongly associated with granulomatous disease, autoimmune disorders, lymphoid hyperplasia and portal hypertension. CVID patients with splenomegaly have significant peripheral lymphopenia ($p=0.001$), significantly fewer peripheral class-switched memory B cells (smBs) ($p=0.001$), CD4+ T lymphocytes ($p=0.001$), NK ($p=0.0001$) and dendritic cells ($p\leq0.01$), and significantly more circulating CD4+ and CD8+ ($p=0.00001$) T-cell-subset activation ($p=0.00005$), than CVID patients without splenomegaly. Examination of splenectomy impact on circulating lymphocyte-subset distributions demonstrated the drastically enhanced total circulating-lymphocyte count post-splenectomy, predominantly B lymphocytes and CD8+ T cells. However, splenectomy did not change B-cell distribution, with smBs remaining persistently low, in contrast to complete inversion of the circulating T-cell composition, with reversal of the CD4+/CD8+ ratio suggesting that amplification of the CD8+ T-cell compartment is a CVID characteristic in patients with splenomegaly. Our results highlight this CD8+ amplification in splenomegaly–CVID patients which might be explained by a homing effect to the spleen and/or possible chronic virus replication, which in turn could induce T-cell expansions.

Conclusion CVID patients with lymphopenia and splenomegaly should not be thought to have combined immune deficiency, but rather true CVID, as their lymphopenia might suggest lymphocyte trapping in the spleen.

Introduction

Common variable immunodeficiency disease (CVID) is a highly heterogeneous group of B-cell-deficiency syndromes, all characterized by low serum immunoglobulins (Igs), impaired antibody responses and recurrent sinopulmonary bacterial infections [1, 2]. Other widely described major clinical manifestations are chronic enteropathy, benign or malignant lymphoproliferation, granulomatous disease and autoimmune disorders, including autoimmune cytopenia [3–6]. At present, CVID patients are differentiated according to the European classification based on flow-cytometry quantification of class-switched memory B cells (smBs: $CD27^{+}IgD^{-}$) and immature circulating B cells ($CD19^{hi}CD21^{-}CD38^{-}$) [7]. The results of the multicenter EUROclass study (303 CVID patients) suggested a close relationship among fewer smBs, more immature B cells and splenomegaly in CVID patients [8].

Splenomegaly is frequent in CVID. For example, based on the European Society for Immunodeficiencies database that includes 2212 CVID patients, 902 with clinical data, 226 (25%) of them had splenomegaly

[6]. It is associated with autoimmune disorders, granulomatous diseases, liver disease and portal hypertension. In addition to higher percentages of $CD21^{low}$ B cells and lower percentages of smBs, we and others demonstrated a significant association between splenomegaly and expansion of circulating $CD8^{+}HLA\text{-}DR^{+}$ T lymphocytes with an effector phenotype in a subset of CVID patients [9–11].

Some CVID patients require splenectomy, intended to cure autoimmune cytopenia for example. To avoid the enhanced risk of severe infections, replacement therapy with intravenous Igs must be rigorously monitored [12]. However, splenectomy consequences on the immune-cell profile of CVID patients have never been studied.

To better understand the spleen's involvement in the immune-system alterations seen in CVID patients, we examined the post-splenectomy distributions and numbers of peripheral lymphocyte subsets. First, CVID patients with splenomegaly and without were compared to evaluate splenomegaly's impact on the immune-cell profiles of these patients. Second, we thoroughly examined the immune-cell phenotypes pre- and post-splenectomy of 11 CVID patients, with flow-cytometry analysis of immune-cell populations in the excised spleens of 8 of them.

Methods

CVID Cohort

Eighty-nine patients were included in this study after giving informed consent: 79 followed in our facility and 10 others from the DEFI cohort, a French national cohort of adults with primary hypogammaglobulinemia that included 313 with CVID [5], added because they had been splenectomized. Among the 79 patients from the University of Bordeaux, 70 had been enrolled in the ALTADIH Cohort (approved by the Bordeaux University Institutional Review Board on December 20, 2006, no. 2.04.2007) and 33 were also DEFI-cohort participants.

Standard criteria [1] were always applied to diagnose CVID, specifically requiring: 1) low serum IgG level < 5 g/L, combined with low IgM- and/or IgA-isotype concentrations < 0.4 g/L or < 0.7 g/L, respectively; 2) poor antibody responses to immunization or infection, and 3) exclusion of other defined forms of primary and secondary hypogammaglobulinemias. Splenomegaly was diagnosed when the spleen was > 13-cm long on computed-tomography scans. According to the absence or the presence of splenomegaly, 2 patient groups were defined according to splenomegaly presence ($n = 48$) or absence ($n = 41$). About half (21/41) of the splenomegaly-positive patients underwent splenectomy; among them, 11 had complete circulating immune-cell phenotyping pre- and post-splenectomy (minimal follow-up: 3 years) and 10 had circulating immune-cell phenotyping only post-splenectomy (either it had not been done pre-splenectomy or could not be retrieved). Eight splenectomized-CVID patients regularly followed at the Bordeaux University Hospital for several years had repeated blood flow-cytometry analyses during the years post-splenectomy (minimum 3 years for all patients, 5 years for 7, 8 years for 5 and maximum 14 years for 2);

those values enabled longitudinal analysis of their lymphocyte subsets. At the time of phenotyping, none of the patients had evidence of acute infection.

Demographic, clinical and biological information from every patient was collected prospectively.

Spleen Preparation

Excised spleens were obtained from 8 CVID patients within the hour following scheduled surgery. We also studied 23 spleens removed from adults after abdominal trauma (14 males and 9 females, median age 34 [range 18–68] years) that served as controls. Spleen samples were analyzed by flow cytometry using a FC500 flow cytometer from Beckman-Coulter. Briefly, sterile spleen tissues in RPMI-1640 medium were mechanically dissociated in a glass tissue homogenizer, then filtered through a 100-µm nylon strainer. The collected cell suspension was incubated for 10 minutes in a hemolytic solution (150 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM ethylenediaminetetraacetic acid) at room temperature to remove red blood cells. The remaining cells were then washed in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and filtered again before cell-surface-determinant labeling. Trypan-blue-dye exclusion assessed cell viability that was usually ∼90%. Samples were then aliquoted for flow-cytometry analyses.

Flow-Cytometry Analyses

Peripheral blood samples were collected to phenotype cells of CVID patients' receiving Ig replacement (drawn before infusion) or not. Only one such evaluation was available per patient, except for the 9 who were splenectomized in Bordeaux and had several sequential phenotype analyses during follow-up. Flow-cytometry analyses were run within the 24 hours following blood withdrawal. Unless specified, all monoclonal antibodies were purchased from Beckman-Coulter (Brea, CA) whose catalog references are given below.

The following panels of monoclonal antibodies (mAbs) (reference) were used: for B-cell differentiation: anti-CD19 (A07766), -CD27 (6607107), -CD21 (PN IMU473U) and -surface (s)IgD (736000); for differentiated $CD8^+$ T cells: anti-CD8 (A07756), -CD45RA (PN IM 271U), -CD27 (6607107); for human leukocyte antigen (HLA)-DR T-lymphocyte activation markers: anti-CD45, -CD3, --CD4 and CD8 (6607013) - HLA-DR (PNA40579); for $CD25^+$ (IM2646) T-lymphocyte activation markers: anti-CD3, - CD4 and - CD8 (6607013).

The following mAb (reference no.) panels were used to label: regulatory T cells (Tregs): anti-CD4 (A07750), -CD25 (IM2646) -CD127 (PN IM1980U); natural killer (NK)/B cells: anti-CD45, -CD56, -CD19 and -CD3 (6607073), and -CD16 (A07766); myeloid (mDCs) and plasmacytoid dendritic cells (pDCs), respectively, the Beckman-Coulter kit (A23413 and A23416); T cells expressing the gamma/delta T-cell receptor (γ/δ TCR): anti-Vdelta (Vδ2; (PN IM1464) and anti-panδ (PN IM1418U). T- and B-cell subpopulation counts were obtained using the fluorescent microbeads kit from Beckman-Coulter (7547053) following lysis (Versalyse; ref: A09777) without any washing according to the manufacturer's recommendations.

For the 10 splenectomized-CVID patients from the DEFI cohort, immunological data were obtained as described previously and were available only post-splenectomy [13].

Results are expressed as percentages of total circulating lymphocytes, or $CD19^+$, $CD3^+$, $CD4^+$ or $CD8^+$ lymphocytes as appropriate, and as absolute counts in cells/mm^3. DCs are an exception; their numbers are expressed per mL of blood, derived by calculations based on the leukocyte count.

Statistical Analyses

Non-parametric tests were used because neither distribution normality nor homoscedasticity was verified. Descriptive analysis values are expressed as median (25th − 75th percentiles) values. Mann–Whitney *U*-test, with a level of significance of $p = 0.05$, was used to compare between-group median percentages and absolute cell counts. The tests were computed with Statistica Inc. software (Statsoft, Tucson, AZ).

RESULTS

Patients

Sociodemographic and clinical characteristics

The 89 CVID patients (median age: 44 (25th − 75th percentiles: 30–56) years; 38 men and 51 women) included were grouped according to their splenomegaly status. Baseline epidemiological and clinical characteristics at diagnosis are reported in Table 1: 51 were women; their median age was 44 (25th − 75th percentiles: 30–56) years. Patients with splenomegaly were younger than those without but the difference was not significant. Their most frequent prior symptom was respiratory tract infections. However, no between-group difference in infectious events was noted. Notably, patients with splenomegaly had significantly more complications, such as lymphoproliferative disorders ($p < 0.0001$), granulomatous disease ($p = 0.009$) and/or hepatic nodular regenerative hyperplasia ($p = 0.04$), while no difference was observed for autoimmune-manifestation rates. More than 80% of the patients were receiving subcutaneous (SC) or intravenous (IV) Ig replacement and, thus, more frequently in patients with splenomegaly.

Table 1
Baseline characteristics of 89 CVID patients according to splenomegaly absence or presence.

Characteristic	No splenomegaly ($n = 41$)*	Splenomegaly present ($n = 48$)
Sociodemographic		
Female	21 (51.2)	30 (62.5)
Median age, years	42 [28–54]	46 [32.5–59]
Clinical		
Otitis, sinusitis, nasopharyngitis, polyposis	31 (75.6)	34 (70.8)
Bronchitis, pneumonia	35 (85.4)	36 (75)
Bronchiectasis	14 (34.1)	12 (25)
Acute diarrhea	4 (9. 8)	6 (12.5)
Chronic diarrhea	8 (19.5)	9 (18.8)
Giardiasis	2 (4.8)	3 (6.3)
Villous atrophy	7 (17.1)	3 (6.3)
Chronic inflammatory intestinal disease	3 (7.3)	2 (4.2)
Autoimmune disease(s)†	16 (39.0)	8 (16.7)
Granulomatous disease‡	13 (31.7)§	0
Lymphoproliferative disorders	24 (58.5)§	5 (10.4)
Hepatic nodular regenerative	8 (19.5)¶	0
Treatment SC or IV Ig substitution	40 (97.6)	41 (85.4)

Results are expressed as n (%) or median [25th – 75th percentiles]. *Twenty-one of these patients will be splenectomized. †Autoimmune anemia, autoimmune neutropenia, autoimmune thrombocytopenia, Hashimoto's thyroiditis, Goujerot–Sjögren's syndrome, autoimmune diabetes, systemic lupus. ‡Histologically proven CVID. §$p < 0.0001$; ¶$p < 0.05$.

CVID-patient classification according to EUROclass

Application of EUROclass distinguished 8/89 (9%) with ≤1% B cells (B^- patients), 3 with splenomegaly and 5 without, and 81 patients with higher percentages of B cells (83% B^+). smB status could not be evaluated for 3 patients (data not available). $B19^+$ patients were divided as follows: 34 (38.20%) with severe smB deficiency (≤ 2% B cells, smB^-) and 44 (49.43%) with > 2% smBs (smB^+). A higher percentage of CVID patients with splenomegaly were smB^- than those without splenomegaly (46% and 29%,

respectively). EUROclass also discriminated patients according to their $CD21^{low}$ B-cell expansion above or below 10% within B cells ($CD21^{low}$ vs. $CD21^{norm}$) [7]. That determination was available only for 57 patients, 34 without splenomegaly and 12 with splenomegaly. Among the 12 CVID patients with splenomegaly for whom the data were available, 3 (25%) were assigned to the $CD21^{low}$ group, as opposed to 11 (32%) without splenomegaly.

Splenomegaly Impact on Circulating Ig Concentrations and Lymphocyte Subsets

Blood Ig concentrations

Supplemental Table S1 shows the CVID patients' median serum IgA, IgM, IgG and IgG-subclass concentrations obtained before replacement therapy, according to splenomegaly status. CVID patients with splenomegaly had significantly lower levels of serum IgA, IgM and IgG than those without (p = 0.0001, 0.008 and 0.009, respectively). All circulating IgG-subclass concentrations were lower in patients with splenomegaly, especially IgG_1 and IgG_2, but significance was not achieved, probably due to the small sample size, as this information was only available for half of the subjects.

Blood-lymphocyte immunophenotyping

Immunophenotyping results are available for only 31 of the 41 patients without splenomegaly (Table 2).

Table 2
Blood immune-cell characteristics in 89 CVID patients according splenomegaly absence or presence pre- and post-splenectomy.

	Unit	No splenomegaly	Splenomegaly present	
			Pre-splenectomy	Post-splenectomy
Number of patients, *n*		48	31	21
TLCs	cells/mm^3	1358.5 (982.5–1948) [305–3655]	897 (652–1419) [153–2915]*	2534 (1439–3564) [654–6579] †
B cells				
CD19$^+$	%	10.4 (6.7–15) [0.1–29.7]	8.8 (4.6–15.2) [1–42.7]	8 (4–12) [0.5–24.4]
CD19$^+$	cells/mm^3	143 (79–217.5) [2–823]	91 (39–135) [1.5–1210]‡	204 (137–268) [3–587]§
CD19$^+$CD27$^-$sIgD$^+$	%	77.8 (59–85.8) [0–99]	87.3 (82.4–92.1) [59.7–96.6]*	89.9 (83–94) [66–97.6]
CD19 + CD27$^+$sIgD$^+$	%	12.1 (5–18.3) [0–73.15]	7.8 (2.8–11.6) [1.1–39.1]	3 (2–5) [0–21]§
CD19$^+$CD27$^+$sIgD$^-$	%	4.91 (1.7–11.5) [0–33.7]	1.4 (0.6–3.3) [0.1–7.7]*	1.3 (0.2–4) [0–10.7]
CD19$^+$CD27$^-$sIgD$^-$	%	2.4 (1.4–3.4) [0–24.7]	1.6 (0.8–2.8) [0–8.4]	4.5 (2–8) [0.4–12]£
T cells				
CD3$^+$	% TCL	76.8 (71–84.0) [58.4–95.6]	79.7 (73–86.8) [49.3–95.5]	75 (68–86) [47–94]
CD3$^+$	cells/mm^3	1036.5 (728–1613.5) [204–2697]	727 (510–1041) [113–2082]‡	1820 (1319–2431) [615–5658] †
CD4$^+$	% TCL	47.3 (40.4–53.7) [22.8–70.5]	45.4 (34.8–53.7) [14.7–76.5]	31 (22.4–36) [12–50.1] †
CD4$^+$	cells/mm^3	645.5 (425.5–988) [87–1746]	387 (292–560) [90–1105]*	660 (532–907) [150–2237] †

For patients without and with splenomegaly, results are expressed as medians (25th – 75th percentiles) and [range]. Their values were compared with the non-parametric Mann–Whitney *U*-test: ‡$p \leq 0.01$ vs. no splenomegaly; ***$p \leq 0.001$ vs. no splenomegaly. †$p \leq 0.00001$ vs. pre-splenectomy. §$p \leq 0.01$ vs. pre-splenectomy; other between-group comparisons were not statistically significant. TCL, total circulating lymphocytes; sIgD, surface IgD; Tregs, regulatory T cells.

	Unit	No splenomegaly	Splenomegaly present	
$CD8^+$	% TCL	24.8 (19.9–30.9) [6.3–58.9]	28.2 (19–35.8) [6.4–56.9]	42 (27.8–53.8) [17.2–65] †
$CD8^+$	cells/mm^3	305 (195.5–584.5) [71–1000]	219 (135–402) [21–906]	774 (541–1647) [191–3618] †
$CD3^+CD4^-CD8^-$	% TCL	3.4 (2.6–5.2) [0.7–11.3]	3.8 (2.5–5.4) [0.7–9.1]	3.2 (1.8–7.8) [0.7–14.8]
$CD3^+CD4^-CD8^-$	cells/mm^3	57 (28–78) [4–176]	31 (16–52) [1–136]‡	109 (36–193) [9–287]
γ/δ-TCR-expressing cells	cells/mm^3	29 (17–63) [4–111] (n = 35)	21 (11–49) [0–126] (*n* = 17)	114 (38–170) [6–368]§
Activated T cells				
HLA-DR				
$CD3^+HLA^-DR^+$	% CD3+	8.6 (5.2–14.6) [1.2–37.1]	24.9 (9.1–40) [6.1–61.7]*	30.2 (16.8–36.6) [9–57.4]
$CD4^+HLA^-DR^+$	% CD4+	7.7 (4.4–12.8) [1.1–54.6]	23 (10.4–44.2) [4.7–72.6]*	23.3 (14.0–34) [9.8–54.6]
$CD8^+HLA^-DR^+$	% CD8+	20.4 (10.4–33.7) [1.5–76]	46.1 (23–70) [14.4–95.4]*	44.83(37.7–65.5) [15.3–89.5]
Natural killer cells				
$CD3^-CD16^+CD56^+$	% TCL	9.7 (5.4–13.8) [1–26.4]	7.5 (3.5–10.4) [1.6–25.4]	10 (4.9–20.5) [3–34.1]
$CD3^-CD16^+CD56^+$	cells/mm^3	133 (83–193) [9–668]	56 (39–93) [12–290]*	171 (94–668) [39–1532] †
Number of patients, *n*		35	19	7
Dendritic cells				
Myeloid	cells/mL	9130 (5110–14283) [1914–38325]	6173 (3796–8582.5) [1162–12973]*	8514 (2312–13154) [2158–18799]

For patients without and with splenomegaly, results are expressed as medians (25th – 75th percentiles) and [range]. Their values were compared with the non-parametric Mann–Whitney *U*-test: ‡$p \leq 0.01$ vs. no splenomegaly; *$p \leq 0.001$ vs. no splenomegaly. †$p \leq 0.00001$ vs. pre-splenectomy. §$p \leq 0.01$ vs. pre-splenectomy; other between-group comparisons were not statistically significant. TCL, total circulating lymphocytes; sIgD, surface IgD; Tregs, regulatory T cells.

	Unit	No splenomegaly	Splenomegaly present	
Plasmacytoid	cells/mL	4429 (2405–9418) [1247–19405]	2575.5 (986.5–4140.5) [417–8291]‡	4157 (2367–23202) [812–26107]
Tregs				
$CD4^+CD25^+CD127^-$	cells/mm^3	38 (22–66) [7–179]	24 (9–42) [4–82‡	27 (11–45) [8–62]

For patients without and with splenomegaly, results are expressed as medians (25th – 75th percentiles) and [range]. Their values were compared with the non-parametric Mann–Whitney *U*-test: ‡$p \leq 0.01$ vs. no splenomegaly; ***p** ≤ 0.001 vs. no splenomegaly. †$p \leq 0.00001$ vs. pre-splenectomy. §$p \leq 0.01$ vs. pre-splenectomy; other between-group comparisons were not statistically significant. TCL, total circulating lymphocytes; sIgD, surface IgD; Tregs, regulatory T cells.

Circulating CD19 + B cells. Median total circulating-lymphocyte percentages and absolute peripheral B-cell counts (/mm^3) were significantly lower in CVID patients with splenomegaly ($p = 0.001$ and $p = 0.01$, respectively, vs. patients without splenomegaly), but their percentages did not differ between groups. However, their circulating B-cell–subset distributions differed markedly between patients without splenomegaly vs. those with it, respectively: significantly higher smBs ($sIgD^-CD27^+$) (4.9% vs. 1.4%; $p = 0.001$) and lower naïve B cells (IgD^+CD27^-) (77.8% vs. 87.3%; $p = 0.0007$), while the percentages of marginal zone B cells (IgD^+CD27^+) were comparable, albeit lower for patients with splenomegaly.

Blood IgA and IgG concentrations correlated (Spearman's correlation test) significantly with the percentages of circulating smBs ($r = 0.64$ and 0.59; $p \leq 0.0001$, respectively) and inversely with percentages of naïve B cells ($r = -0.56$ and -0.47; $p \leq 0.01$, respectively).

Circulating T cells. The median absolute numbers of circulating $CD3^+$ T-lymphocytes was significantly lower in CVID patients in general, but especially in those with splenomegaly compared to those without, with $CD4^+$ cells contributing significantly more than the $CD8^+$ subset ($p = 0.001$ and 0.12, respectively). No difference in their $CD4^+/CD8^+$ ratios was noted. CVID patients with splenomegaly had also significantly fewer circulating Tregs ($CD4^+CD25^+CD127^-$) ($p = 0.01$ vs. those without splenomegaly). However, the percentages of the different T-lymphocyte subsets did not differ much between CVID groups.

Although based on only 14 patients, median absolute numbers and percentages of circulating $CD27^+CD45RA^+CD8^+$ naïve T cells were significantly lower in CVID patients with splenomegaly than those without (16/mm^3 vs. 56/mm^3; $p = 0.002$, and 6.31% vs. 20%; $p = 0.01$, respectively), but no difference was noted concerning memory T cells (data not shown). For $CD4^+$ T cells, no difference between the 2 groups was found according the memory or naïve phenotype.

NK and DCs. Patients with splenomegaly had significantly lower median circulating NK counts than patients without ($p = 0.0001$), but their percentages were comparable. Median absolute numbers of circulating mDCs and pDCs were also lower in patients with splenomegaly ($p \leq 0.01$ vs. those without).

Activated T cells. HLA-DR expression at the surface of T lymphocytes reflects their activation status and was examined on T-cell subsets in each CVID-patient group. The median percentage of HLA-DR-expressing $CD3^+$ T cells was significantly higher in patients with splenomegaly ($p = 10^{-5}$ vs. those without), as were overall percentages of $CD4^+$ and $CD8^+$ DR^+ T cells ($p = 5\times10^{-5}$ and $p = 10^{-5}$ vs. those without, respectively).

Splenectomy Impact on the Immune-Cell Profile of CVID Patients

Sociodemographic and clinical characteristics

Information was collected from 21 CVID patients (12 males and 9 females) who had been splenectomized and their individual clinical characteristics are reported in **supplemental Table S2**. All had splenomegaly prior to splenectomy. Nineteen had recurrent bacterial respiratory tract infections; only 2 patients remained infection-free. However, all 21 patients had other clinical manifestations including autoimmune disorders (11 idiopathic thrombocytopenic purpura, 4 autoimmune hemolytic anemia, 3 autoimmune neutropenia, and 1 each: erythroblastopenia, Sjögren's syndrome, pernicious anemia or Hashimoto's thyroiditis), organomegaly caused by lymphoid hyperplasia ($n = 18$), chronic granulomatous disease diagnosed from lymph-node biopsies and/or splenectomy ($n = 7$), hepatic nodular regenerative hyperplasia ($n = 7$) or villous atrophia ($n = 4$). The main indications for splenectomy were worsening cytopenia or suspected lymphoma.

Immune-cell phenotypes

Circulating lymphocytes from the 21 splenectomized-CVID patients were phenotyped 12–24 months post-splenectomy. Nine patients' cells were phenotyped several times during follow-up but only values from the first post-splenectomy analysis are reported (Table 2).

Median total circulating-lymphocyte counts rose significantly post-splenectomy ($p = 10^{-5}$ vs. CVID patients with splenomegaly), reaching a higher level than CVID patients without splenomegaly ($p = 0.0009$). Splenectomized patients also had significantly higher median absolute B-cell numbers that with patients with splenomegaly ($p = 0.002$) (Fig. 1A), but median percentages of B-cell subsets changed little, particularly smB percentages did not differ between pre- and post-splenectomy assessments. However, the median B-lymphocyte increase concerned mainly double-negative IgD^-CD27- B cells, while the median percentage of transitional IgD^+CD27^+ lymphocytes declined significantly post-splenectomy (from 7.8–3%; $p = 0.01$).

Post-splenectomy lymphocytosis was mostly explained by expansion of the $CD8^+$ T-cell compartment, which was more pronounced than that of the $CD4^+$ T-cell compartment ($p = 5\times10^{-6}$, $p = 0.001$ vs. patients with splenomegaly and $p = 6\times10^{-5}$, $p = 0.69$ vs. patients without splenomegaly, respectively), leading to an inversion of the $CD4^+/CD8^+$ ratio after splenectomy (Fig. 1B). While the median number of $CD8^+$ T cells increased dramatically post-splenectomy, the median percentages of the different $CD8^+$ T-cell subsets did not change (data not shown). Moreover, we observed marked expansion of the γ/δ T-cell subset post-splenectomy compared to CVID patients with splenomegaly, exceeding the values observed in CVID patients without splenomegaly ($p = 0.001$ and $p = 0.003$, respectively). Concerning the Tregs, no between-group differences were seen, while median absolute peripheral NK-cell numbers rose significantly in splenectomized patients ($p = 0.0001$ vs. patients with splenomegaly), reaching the level observed in CVID patients without splenomegaly. DC levels were also elevated post-splenectomy but returned to initial values observed in patients without splenomegaly (no significant between-group difference).

Splenectomy did not affect T-cell–compartment activation, as assessed by HLA-DR expression on the T-cell surface. However, the activation levels of $CD4^+$ and $CD8^+$ T-cell subsets remained significantly higher than those of CVID patients without splenomegaly ($p = 10^{-6}$ and 2×10^{-6}, respectively).

Analysis of Lymphocyte Subsets percentages in the Spleens of 8 CVID Patients and Controls

While normal spleens contain more B cells than T cells, we observed the opposite in CVID spleens (**supplemental Table S3).** Compared to normal spleens, CVID spleens contained markedly fewer median $CD19^+$ B cells [$p = 0.0001$) and significantly more median T cells ($p = 0.0003$) (Fig. 2A) with $CD8^+$ cells contributing significantly more than the $CD4^+$ subset ($p = 0.0001$ and 0.01, respectively). The $CD8^+$ T-cell population was more activated than in control spleens, but overall T-cell activation did not differ between populations (Fig. 2B). We did not observe any differences between the 2 splenic populations regarding the percentages of Vγ/δ TCR-expressing T-cell subsets and NK cells. In contrast, DCs were all but absent in CVID spleens, which could be consistent with their non-circulatory nature, while pDCs were the only subpopulation present in normal spleens.

The percentages of lymphocyte subsets in the spleens and peripheral blood of the 8 CVID splenectomized patients were compared. No differences were observed between the 2 compartments in their subset distributions and T-cell–activation levels for a given patient (data not shown).

Evolution of Lymphocyte Populations Post-Splenectomy

Circulating immune-cell phenotyping was repeated in the same center during post-splenectomy follow-up of 8 CVID splenectomized patients. Figure 3 shows their evolutions before and up to 8 years post-splenectomy. Lymphocytosis increased dramatically during the first-year post-splenectomy and, thereafter, the median total peripheral lymphocyte count remained globally stable over time. That

expansion concerned all lymphocyte subsets but was most marked for $CD8^+$ T cells with inversion of the $CD4^+/CD8^+$ ratio maintained throughout follow-up. The median absolute total peripheral lymphocyte count spiked at year 5, probably linked to random and transient $CD8^+$ T-cell mobilization that varies individually. T-cell activation tended to decline over time. Blood DCs—similarly for mDCs and pDCs—also decreased progressively during follow-up (data not shown).

Discussion

We first confirmed previous findings showing the strong association of splenomegaly with granulomatous disease and autoimmune disorders [6, 10, 14]. We also found a significant link with lymphoid hyperplasia and portal hypertension due to hepatic nodular hyperplasia observed in 9 patients with splenomegaly. Patients with splenomegaly tended to have lower percentages of total B cells but the distribution of B-cell subsets was significantly perturbed with fewer smBs and marginal zone B cells, and higher percentages of naïve B cells, as observed in the EUROclass trial [7]. They also suffered from more profound hypogammaglobulinemia for all Ig classes, which correlated with their rarified smBs. Splenomegaly was associated with significantly fewer $CD4^+$ T lymphocytes and Tregs, but their $CD4^+/CD8^+$ ratios did not differ from those of patients without splenomegaly. $CD4^+$ and $CD8^+$ T-cell subsets were significantly more activated when splenomegaly was present, while peripheral NK and DC counts were significantly lower.

We then analyzed the impact of the splenectomy on the distributions of circulating-lymphocyte subsets hypothesizing that splenectomy might explain some of their phenotypic characteristics in CVID patients. Total blood-lymphocyte counts increased dramatically post-splenectomy, predominantly attributable to expansion of B cells and $CD8^+$ T cells.

Our main finding is the significant lymphopenia in patients with splenomegaly that normalized post-splenectomy. That observation is fundamental because lymphopenia is an exclusion criterion for CVID diagnosis, leading patients to be diagnosed with “Late onset combined immunodeficiency” (LOCID) [15]. Among our 31 patients with splenomegaly, 18 had $CD4^+$ T-cell counts < $200/mm^3$, thereby satisfying the LOCID definition of a combined deficiency. However, post-splenectomy, the 4 with $CD4^+$ T lymphopenia below that threshold all saw their lymphopenia corrected, thereby excluding the diagnosis of combined deficiency and returning to the definition of CVID. Patients thought to have a combined deficiency because of lymphopenia might not have true lymphopenia because of splenomegaly. With this knowledge, we believe that CVID-diagnosis should still be attributed for patients with splenomegaly despite lymphopenia.

However, the B-cell distribution remained unchanged by splenectomy, with persistence of fewer smBs, suggesting that this deficiency is intrinsic to CVID and not related to smB-trapping in the spleen. In contrast, splenectomy led to notable changes of the circulating T-cell composition, with inversion of the $CD4^+/CD8^+$ ratio that usually declines post-splenectomy in normal individuals [16], suggesting that $CD8^+$

T-cell–compartment amplification is a characteristic specific to CVID patients with splenomegaly. That notion was confirmed by the persistent lymphocyte expansion over time (Fig. 3) and is therefore not influenced by intercurrent factors such as infections. Our results highlight the importance of this $CD8^+$ amplification in such patients, which could be explained as a homing effect to the spleen and/or possible chronic viral replication, which in turn could induce T-cell expansions.

We previously showed that CVID patients, notably those with complications, had oligoclonal expansions of their $CD8^+$ T-cells—not resulting from acute clinical infectious events—reflecting a stable chronic phenotypic T-cell pattern, possibly in relationship with chronic viral replication, facilitated by the immunodepression [11]. However, this oligoclonal expansion was mainly observed in CVID patients with splenomegaly, a finding that suggests a trapping process in the spleen when it is enlarged. Pertinently, that the percentages of the different $CD8^+$ T-cell subsets were unaffected by splenectomy compared to pre-splenectomy values is a strong argument for a homing phenomenon.

To explore that hypothesis, we examined the cellular compositions of spleens excised from 8 CVID patients and compared them to those of 23 controls. The distributions of lymphocyte subsets in normal spleens differed markedly from those in peripheral blood. Langeveld et al. analyzed the splenic lymphocyte compositions of 16 organ-transplant donors [17]. Compared to their peripheral blood, their spleens contained significantly more B cells than T cells, more $CD8^+$ T cells and less $CD4^+$ T cells with an inverse $CD4^+/CD8^+$ ratio, a higher percentage of activated T cells and more $CD8^+$ cytotoxic T cells. The same cellular distribution pattern was observed in CVID patients' spleens but highly amplified; compared to normal spleens, although the CVID spleens contained few B cells, $CD8^+$ activated-T cells were expanded, suggesting trapping of these cells in these patients' spleens.

Further studies are needed to elucidate why $CD8^+$ T cells are retained in the spleen for longer periods in CVID patients. Their egress from the spleen into blood requires a sphingosine-1-phosphate (S1P) concentration differential at the site of transmigration [18], i.e., high in blood and lymph compared to the interstitial fluid of lymphoid organs. Moreover, S1P receptor-1 (S1PR1) expression on lymphocytes was found to be critical for lymphocyte egress from the thymus and secondary lymphoid organs. Egress modulation is regulated according to the S1P-concentration–S1PR1-expression balance. It is under the influence of several markers, e.g., the activation marker CD69, which limits exit during an immune response by inhibiting the S1PR1 function [19], or factors controlling S1PR1 transcriptional regulation. In this context, CD69 and S1PR1 expressions on T lymphocytes of CVID patients with splenomegaly warrant further investigation. Similarly, another hypothesis to examine would be the influence of chemokines on T-cell–migratory activity in the spleen, particularly the stromal cell-derived factor-1 (SDF-1)/CXCR4 (the α-chemokine receptor specific to SDF-1) axis, whose dysregulations have been implicated in idiopathic $CD4^+$ T-cell lymphocytopenia [20]. An additional avenue of exploration would be potential T-lymphocyte recruitment via aberrant SDF-1 expression by the spleens of CVID patients that would act as a transient reservoir.

Our study obviously has some limitations. First, our sample size is small. However, recourse to splenectomy is not common for CVID patients because of its associated increased infectious risk; thus, we have a meaningful number of splenectomized patients whose phenotypes could be compared before vs. several times post-splenectomy, which has never been reported previously. Second, the numbers of phenotypes obtained for each patient and their frequencies are quite heterogeneous. Nonetheless, phenotyping was repeated at least 3 times over the 3 years after splenectomy for the 8 splenectomized patients followed post-surgery, supporting the good reliability of those results. Finally, the lack of pre-splenectomy phenotyping for 10 of the 21 splenectomized patients represents a data-analysis bias.

In conclusion, splenectomizing CVID patients with splenomegaly restores the absolute circulating lymphocyte count, suggesting that these patients do not have a true combined deficiency and meet CVID criteria.

Declarations

Author Contributions J-FV and CF enrolled CVID patients in the cohort and collected clinical data. PB and J-FM ran the flow-cytometry analyses. MP conducted the histological analyses and tissue immunohistochemical labeling. J-FV computed the statistical analyses of the data. J-FV, J-FM and CF wrote the paper. All authors approved the final version for submission.

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Data Availability. Not applicable.

Ethics Approval and Consent to Participate. CVID patients were enrolled in the ALTADIH Cohort which was approved by the Bordeaux University Institutional Review Board on December 20, 2006 (no. 2.04.2007). Each CVID patient gave informed written consent before participating in the study.

Consent for Publication. The patient consent form contains permission to publish data without an identifier.

Conflict of Interest The authors declare no conflicts of interest.

Supplementary Information The online version contains supplementary material.

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Figures

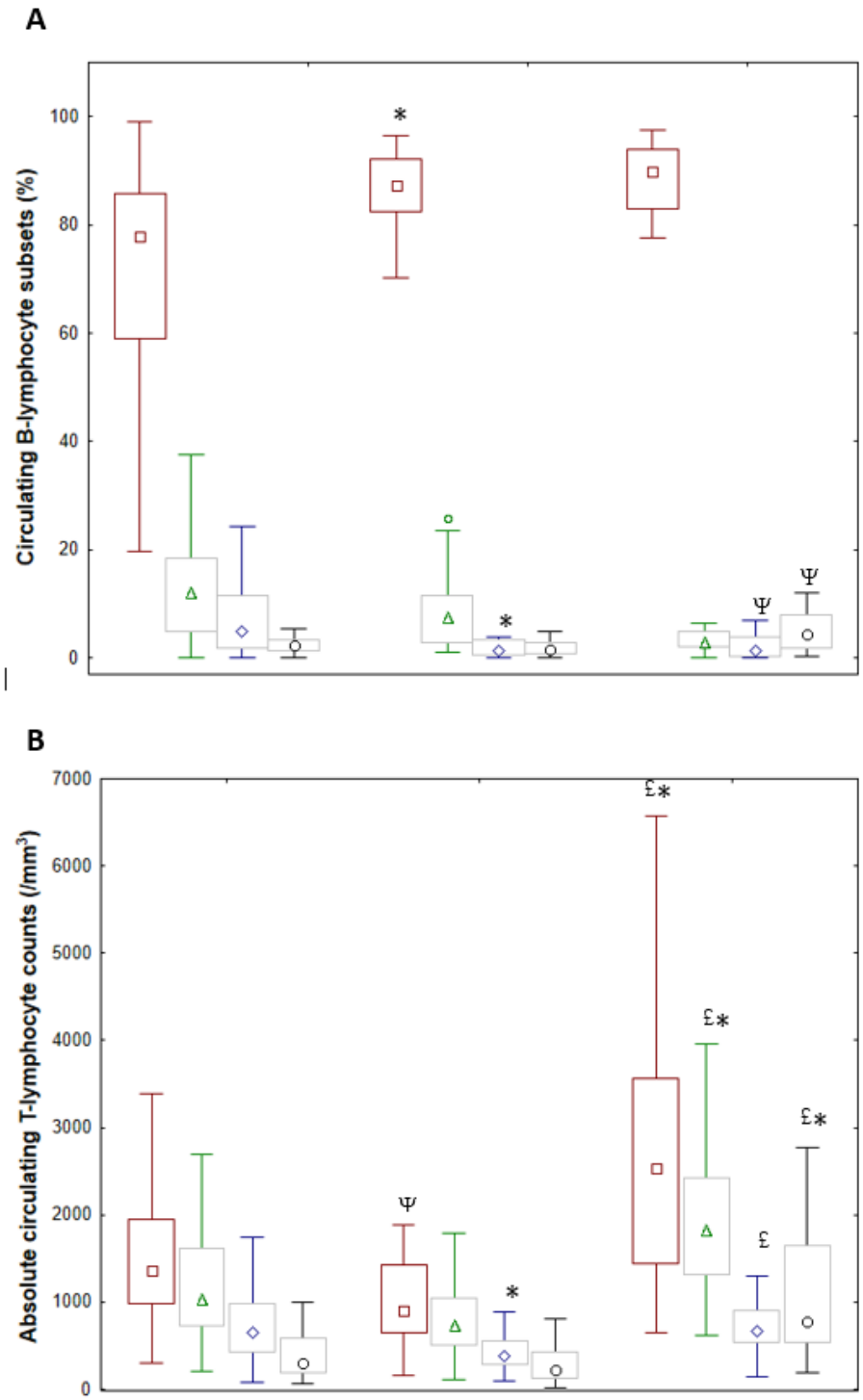

Figure 1

Flow-cytometry analyses of circulating lymphocytes from CVID patients—48 without splenomegaly, 31 with splenomegaly and 21 splenectomized—illustrated as box plots. The symbols inside the boxes are medians, lower and upper limits of the boxes are the 25th and 75th percentiles, and T-bars represent the 10th and 90th percentiles. Statistically significant (non-parametric Mann–Whitney *U*-test) between-group comparisons are indicated. **A.** Percentages of circulating B-cell subsets. □, % IgD^{+}CD27^{-}; Δ, %

IgD^{+}CD27^{+}; ◊, % IgD^{-}CD27^{+}; and ●, % IgD^{-}CD27^{-}. *$p \leq$ 0.001 vs. patients without splenomegaly; $^{Y}p \leq$ 0.01 vs. patients with splenomegaly. **B.** Absolute total circulating lymphocyte and T-cell-subset counts (CD3^{+}, CD4^{+}, CD8^{+}). □, total lymphocytes; Δ, CD3^{+} T cells; ◊, CD4^{+} T cells; and ●, CD8^{+} T cells) $^{Y}p \leq 0.01$ vs. patients without splenomegaly; ***p**≤0.001 vs. patients without splenomegaly; $^{£}p \leq 0.00001$ vs. patients with splenomegaly.

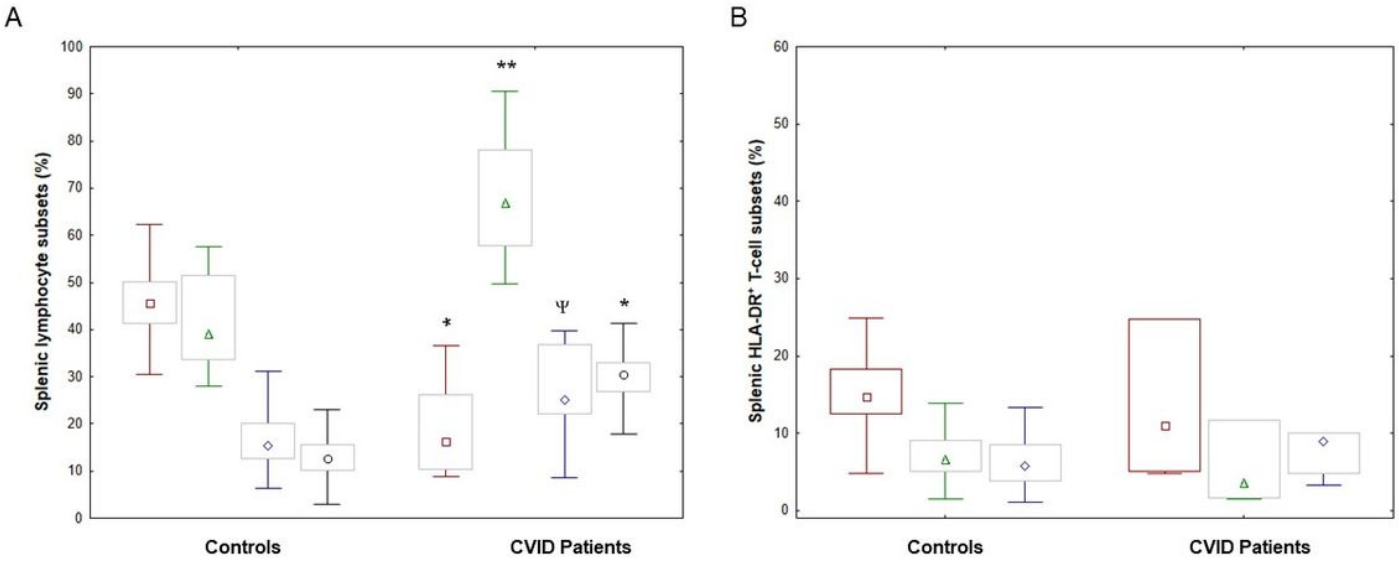

Figure 2

Flow-cytometry analyses of lymphocyte subsets in the excised spleens of 8 splenectomized-CVID patients and 23 adult abdominal trauma patients (controls) illustrated as box plots. The symbols inside the boxes are medians, lower and upper limits of the boxes are the 25th and 75th percentiles, and T-bars represent the 10th and 90th percentiles. Statistically significant (non-parametric Mann–Whitney *U*-test) between-group comparisons are indicated. **A.** Percentages of lymphocyte subsets: □, % CD19^{+} B cells; Δ, % CD3^{+} T cells; ◊, % CD4^{+} T cells; and ●, % CD8^{+} T cells. *p=0.0001 vs. controls; **p =0.0003 vs. controls; ^{Y} p=0.01 vs. controls. **B**. Percentages of HLA-DR^{+} T cells. □, HLA-DR^{+}CD3^{+}; Δ, HLA–DR^{+}CD4^{+}; and ◊, HLA-DR^{+} CD8^{+}. No statistically significant differences were observed between the 2 populations.

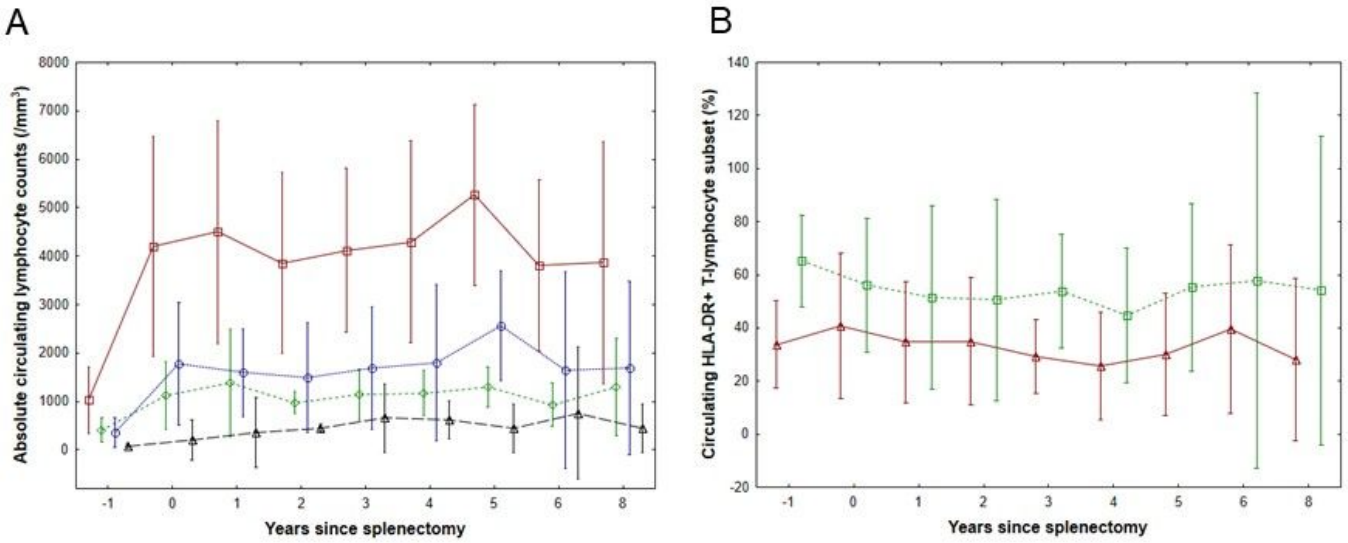

Figure 3

A. Longitudinal flow-cytometry analyses of absolute lymphocyte and subset counts in 8 CVID patients pre- and post-splenectomy. □, total lymphocytes; Δ, B cells; ◊, $CD4^+$ T cells; and ●, $CD8^+$ T cells; are the medians and vertical bars represent the 10^{th} and 90^{th} percentiles. **B**. Longitudinal flow-cytometry analyses of $CD4^+$ (Δ) and $CD8^+$ (□) HLA-DR^+ T-lymphocyte/cell-subset percentages from 8 CIVD patients pre- and post-splenectomy. The squares and triangles represent the medians, while the bars outside the medians correspond to the 10 and 90 percentiles.

Supplementary Files

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- SupplementaryTable1.docx
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