Noninfectious complications in patients with pediatric-onset common variable immunodeficiency correlated with defects in somatic hypermutation but not in class-switch recombination

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Background: Common variable immunodeficiency (CVID) is a heterogeneous syndrome characterized by impaired immunoglobulin production and usually presents with a normal quantity of peripheral B cells. Most attempts aiming to classify these patients have mainly been focused on T- or B-cell phenotypes and their ability to produce protective antibodies, but it is still a major challenge to find a suitable classification that includes the clinical and immunologic heterogeneity of these patients.
Objective: In this study we evaluated the late stages of B-cell differentiation in a heterogeneous population of patients with pediatric-onset CVID to clinically correlate and assess their ability to perform somatic hypermutation (SHM), class-switch recombination (CSR), or both.
Methods: We performed a previously reported assay, the restriction enzyme hotspot mutation assay (IgκREHMA), to evaluate *in vivo* SHM status. We amplified switch regions from genomic DNA to investigate the quality of the double-strand break repairs in the class-switch recombination process *in vivo*. We also tested the ability to generate immunoglobulin germline and circle transcripts and to upregulate the activation-induced cytidine deaminase gene through *in vitro* T-dependent and T-independent stimuli.
Results: Our results showed that patients could be classified into 2 groups according to their degree of SHM alteration. This stratification showed a significant association between patients of group A, severe alteration, and the presence of noninfectious complications. Additionally, 60% of patients presented with increased microhomology use at switched regions. *In vitro* activation revealed that patients with CVID behaved heterogeneously in terms of responsiveness to T-dependent stimuli.
Conclusions: The correlation between noninfectious complications and SHM could be an important tool for physicians to further characterize patients with CVID. This categorization would help to improve elucidation of the complex mechanisms involved in B-cell differentiation pathways. (J Allergy Clin Immunol 2017;139:913-22.)

Key words: *Common variable immunodeficiency, somatic hypermutation, class-switch recombination, hypermutation, pediatric, switch*

The hypogammaglobulinemia of at least 2 immunoglobulin isotypes characterizes the heterogeneous group of the primary immunodeficiency disorder known as common variable immunodeficiency (CVID; OMIM #240500).[1-5] These patients are particularly susceptible to recurrent infections of the respiratory tract, as well as the gastrointestinal system, and manifest noninfectious complications, such as autoimmunity, gastrointestinal disorders, and lymphoproliferation.[1,5]

Despite the rarity of this condition, CVID is the most common immunodeficiency of clinical significance whose main immunologic defect, the failure of immunoglobulin production, is usually associated with a normal peripheral B-cell counts.[1,5] Genetic defects associated with survival, differentiation, and/or immune interactions of B cells have been proposed to be responsible for the pathophysiology of the disease. Several attempts aiming to classify patients with CVID have mainly focused on T- or B-cell phenotypes and their ability to produce protective antibodies,[2,6,7] but it is still a major challenge to find a suitable classification that includes the clinical and immunologic heterogeneity of these patients and their variable response to treatment. The plasma IgG level has poor predictive value in patients with CVID because patients with slightly decreased IgG levels can be highly susceptible to infections and benefit from replacement with intravenous gammaglobulin therapy.[8] Nevertheless, the molecular mechanisms underlying the maturation of the antibody response triggered by natural infections and vaccines have been poorly investigated in these patients.

During somatic hypermutation (SHM), point mutations are introduced into the V region of immunoglobulin genes (both IgH as IgL), increasing antibody affinity for the antigen, and this process is essential for the generation of long-lived plasma and memory B cells.[9-11] Failure in the affinity maturation process of

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Abbreviations used
AICDA: Activation-induced cytidine deaminase
Bcl-6: B-cell lymphoma 6
CSR: Class-switch recombination
CT: Circle transcript
CVID: Common variable immunodeficiency
DSB: Double-strand break
GLT: Germline transcript
IgκREHMA: Restriction enzyme hotspot mutation assay
NHEJ: Nonhomologous end joining
SHM: Somatic hypermutation
TD: T-dependent
TI: T-independent

all isotypes of antibodies could underlie a qualitative humoral immunodeficiency. In previous reports impaired SHM was associated with a high frequency of severe respiratory tract infections in a cohort of mainly adult patients with CVID.[12] In addition, antibody response maturation also requires the class-switch recombination (CSR) process, in which the Cμ region is replaced by a downstream immunoglobulin CH gene, resulting in a change from IgM to IgG, IgA, or IgE, a mechanism involving S regions located upstream of each CH except Cδ.[13]

Both SHM and CSR are dependent on activation-induced cytidine deaminase (AICDA) function, an enzyme expressed in antigen-activated B cells that generates high-affinity antibodies and antibody CSR, respectively.[14] To achieve CSR, AICDA generates double-strand breaks (DSBs) mainly repaired through a nonhomologous end-joining (NHEJ) mechanism[15] by using little or no sequence homology. The NHEJ machinery requires a large number of factors (eg, Ku70/Ku80, DNA-PKcs, DNA ligase IV, Artemis and Cernunnos),[16-20] and defects in this mechanism lead to alternative pathways (eg, use of microhomology) in an attempt to repair DSBs at the S junctions, as shown in patients with primary immunodeficiencies involving defects in NHEJ genes.[21,22]

In this study we evaluated the capacity to perform SHM and CSR *ex vivo* and *in vitro* in a population of patients with pediatric-onset CVID whose immunologic and clinical data were previously reported,[23] allowing us to propose 3 different subsets of patients with CVID.

METHODS

Patients and control subjects

This study included 25 unrelated patients with CVID (12 male patients) with a mean age of onset of symptoms of 5.6 years (range, 1-14 years) and a mean age of diagnosis of 11.3 years (range, 4-16.1 years). Mutations in the *CD40L*, *CD40*, *AICDA*, *UNG*, *PMS2*, *DCLRE1C* (ARTEMIS), and *DNA LIG4* genes were excluded in patients presenting with normal or with increased IgM levels. Patients with decreased peripheral B-cell counts were also excluded from the study. We considered patients as belonging to the smB^+ (switched memory B cells >2%) or smB− (switched memory B cells <2%) subgroups, depending on their B-cell immunophenotype, according to the EUROclass CVID classification.[6] These patients were previously reported (Patients Part B),[23] and the main clinical and immunologic data were summarized in Tables I and II. Informed written consent was obtained from the patient or parental guardian before participation in accordance with the Declaration of Helsinki.

Twenty-two healthy donors matched for age, sex (11 male donors), and ethnic background were included in the study and provided written consent under a separate ethics protocol for healthy donors. Five cord blood samples were included as negative controls in the restriction enzyme hotspot mutation assay (IgκREHMA) and were donated after informed consent was obtained from the delivering women. The research protocol was approved by the internal ethics review board of the Hospital Garrahan.

Total RNA preparation and cDNA synthesis

Total RNA was extracted from PBMCs by using TRIzol reagent (Invitrogen, Carlsbad, Calif), and cDNA was prepared with a first-strand cDNA synthesis kit (Amersham Biosciences, Little Chalfont, United Kingdom), according to the manufacturer's instructions.

RNA from memory and naive B-cell subsets was obtained by using the RNeasy Micro Kit (Qiagen, Hilden, Germany), whereas cDNA was prepared with SuperScript III (Invitrogen), according to the manufacturer's instructions.

Human cell isolation

PBMCs were isolated by means of density centrifugation over a Ficoll-Hypaque Plus (Amersham) gradient. B cells from 7 patients and 4 healthy donors were purified from PBMCs by means of negative selection with the MACS B Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Memory and naive B-cell subsets were purified from total B cells obtained by using the FACSAria Cell Sorter (BD Biosciences, San Jose, Calif) with CD27–fluorescein isothiocyanate, IgD–phycoerythrin, and CD19–phycoerythrin-Cy7 (all from eBioscience, San Diego, Calif). All fractions were obtained with a purity of greater than 95%.

Cell cultures and reagents

Culture of PBMCs or naive B cells from patients with CVID or healthy donors was performed in complete RPMI medium supplemented with 10% (vol/vol) FBS (Gibco, Grand Island, NY). In the T-dependent (TD) stimulation cells were incubated with 500 ng/mL CD40 ligand (CD40L; PeproTech, Rocky Hills, NJ) and 200 U/mL IL-4 (Schering-Plough, Kenilworth, NJ). CD40 was cross-linked with 1 μg/mL mouse 89 mAb (Schering-Plough). Mouse IgG_1 mAb with irrelevant binding activity (Santa Cruz Biotechnology, Dallas, Tex) was used as a control. In the T-independent (TI) stimulation cells were incubated with 5 μg/mL phosphorothioate-modified 5′-tcgtcgttttgtcgttttgtcgtt-3′ oligodeoxynucleotide-2006 (Operon Technologies, Olive Branch, Miss) and 50 ng/mL IL-10 (PeproTech).

RT-PCR and quantitative RT-PCR

Quantification of human Iγ1-Cγ1 or Iα-Cα germline transcripts (GLT), switched Iγ1-Iγ2–Cμ or Iα-Cμ circle transcripts (CTs), AICDA transcript, and glyceraldehyde-3-phosphate dehydrogenase was performed from total RNA extraction in 2- or 4-day cultures with TRIzol (Invitrogen). cDNA was generated by mean of reverse transcription with Superscript II RT (Invitrogen). PCR primers and conditions used for standard or quantitative RT-PCR analysis were performed, as previously reported.[24-26] Results of qRT-PCR were normalized to *ACTB* mRNA and presented as relative expression compared with that of B cells incubated with control antibody.

Characterization of SHM

The IgκREHMA was performed, as previously described.[12]

Characterization of switch recombination junctions

Genomic DNA was purified from peripheral blood cells from patients and healthy donors by using standard methods. Amplification of Sμ-Sα from *in vivo* switched cells was performed by using a previously described nested PCR assay.[21] The PCR-amplified switch fragments were gel purified (GE Healthcare, Fairfield, Conn) and cloned into pGEM-T vector (Invitrogen), and sequence analysis was performed with DNA Sequencing Analysis software (PE Applied Biosystems, Foster City, Calif) on an ABI 3130 (Applied Biosystems). CSR junctions were determined by aligning the

TABLE I. SHM measured by using IgκREHMA and its correlation with clinical and immunologic data

	No.*	IgkREHMA: % SHM†	Switched memory B cells‡	Nonswitched memory B cells§	EUROclass‖	Noninfectious complications	Age at the time of the sample (y)	Immunoglobulin serum levels#		
								IgG	IgA	IgM
Patient group A	1 (27)	2.8	↓ (0%)	↓ (2%)	smB−	SPM	15.8	115	<7	18
	2 (6)	3.3	↓ (0%)	↓ (3%)	smB−		8.2	12	<7	10
	3 (13)	3.5	↓ (0%)	↓ (2%)	smB−	AI (TID)	15.0	62	<7	33
	4 (15)	3.8	↓ (0%)	↓ (2%)	smB−		12.9	220	<7	49
	5 (17)	4.0	↓ (0%)	↓ (7%)	smB−		13.5	193	<7	15
	6 (25)	5.8	↓ (0%)	↓ (2%)	smB−	SPM	15.7	42	<7	10
	7 (10)	8.6	↓ (0%)	↓ (6%)	smB−		10.7	135	24	26
	8 (28)	8.7	↓ (2%)	Norm (15%)	smB−	AI (ITP/AIHA) and SPM	17.3	29	<7	145
	9 (21)	10.7	↓ (1%)	↓ (5%)	smB−		15.1	352	<7	17
	10 (26)	10.8	↓ (0%)	↓ (2%)	smB−	GR and SPM	15.8	162	<7	32
	11 (7)	11.2	↓ (3%)	Norm (9%)	smB+		7.8	302	<7	13
	12 (9)	11.7	↓ (3%)	↑ (19%)	smB+		9.7	293	<7	28
	13 (20)	12.2	↓ (1%)	↓ (2%)	smB−	AI (ITP) and SPM	13.4	391	22	30
	14 (8)	12.6	↓ (1%)	↓ (3%)	smB−		7.2	456	<7	19
	15 (14)	13.1	↓ (0%)	↓ (1%)	smB−		12.7	495	<7	38
	16 (11)	14.8	↓ (2%)	↓ (2%)	smB−		10.9	75	<7	12
Patient group B	17 (22)	18.5	↓ (3%)	↓ (7%)	smB+		15.0	536	<7	43
	18 (18)	19.1	↓ (5%)	Norm (14%)	smB+		13.0	342	<7	18
	19 (23)	19.2	↓ (3%)	Norm (10%)	smB+		14.9	293	<7	25
	20 (5)	19.7	↓ (5%)	↓ (5%)	smB+		5.9	372	21	9
	21 (19)	20.6	↓ (1%)	↓ (5%)	smB−	GR	13.4	315	19	83
	22 (12)	22.1	↓ (2%)	Norm (9%)	smB−		11.1	452	12	36
	23 (4)	25.6	Norm (10%)	↓ (7%)	smB+		4.0	312	15	28
	24 (24)	26.0	↓ (2%)	↓ (0%)	smB−	AI (ITP)	14.9	435	11	33
	25 (16)	50.5	↓ (1%)	↓ (5%)	smB−	AI (ITP)	12.5	462	20	23

	No.	IgkREHMA: mean % SHM†	Mean age at the time of the sample (y)
Healthy donors	22	45.9 ± 9.0 (range, 30.3-63.1)	10.5 (range, 2.2-17.4)
Patients	25	14.5 ± 10.3 (range, 2.8-50.5)	12.3 (range, 4.0-17.3)

‡ and §: ↓, decreased number; ↑, increased number; *Norm*, within normal range.
AI, Autoimmunity; *AIHA*, autoimmune hemolytic anemia; *GR*, granulomas; *ITP*, idiopathic thrombocytopenic purpura; *SPM*, splenomegaly; *TID*, type I diabetes.
*Patient number in a previous report[20] is shown in parentheses.
†Percentage of mutated fraction.
‡Switched memory B cells = $CD19^+CD27^+IgD^-$ cells.
§Nonswitched memory B cells = $CD19^+CD27^+IgD^+$ cells.
‖smB+ indicates less than 2% of switched memory B cells, and smB− indicates 2% or less switched memory B cells.
#Immunoglobulin serum levels at the time of the sample (in milligrams per deciliter).

switched fragment sequences with Sμ (X54713) and Sα1 (L191219) or Sα2 (AF030305). Analysis of microhomology use and mutation patterns at CSR junctions was performed, as described previously.[21,27]

Statistical analysis

Statistical analysis of results was performed with Student 2-tailed *t* tests by using Prism software (GraphPad Sotware, La Jolla, Calif), Spearman 2-tailed tests, or 1-way ANOVA followed by the Dunnett multiple comparison posttest. Frequencies of noninfectious complications among the groups were compared by using the χ^2 test. Other statistical analyses were performed with χ^2 tests with SPSS 15.0 software for Windows (IBM, Armonk, NY).

RESULTS

B cells of patients with CVID carry significantly diminished SHM

To evaluate the SHM levels in peripheral cells from our patients, we performed an assay developed by Andersen et al.[12] This assay is based on cleavage by using a restriction enzyme of a hotspot in the rearranged VκA27 transcripts (IgκREHMA). Briefly, somatic mutations in the restriction sites of the most commonly used light chain gene (κA27) abrogate cutting with a specific enzyme. The variability observed in our healthy control group remained within the levels previously reported (Fig 1, *A* and *B*),[12] which did not seem to be modified by donor age greater than 2 years.

The comparison of our CVID patient group with the healthy donor group revealed a significant difference in mutations of the VκA27 gene ($P < .0001$, Student 2-tailed *t* test; Fig 1, *A* and *B*). At the same time, a heterogeneous behavior in undergoing SHM could be observed in the pediatric population with CVID (Fig 1, *A* and *B*), and this heterogeneity correlates with the percentage of switched memory B cells (Spearman $r = 0.68$, $P = .0002$; Fig 1, *C*) but not with total memory B cells or nonswitched memory B cells (data not shown).

Almost all of the patients (24/25) had decreased SHM values (Table I). Based on these results, a relevant cutoff could optimize the distinction of patients with clinical complications, reduced switched memory B-cell counts, or both. Thus we established 2 subgroups of patients with altered SHM: group A with

TABLE II. Sμ-Sα junction microhomology analysis and their correlation with clinical and immunologic data

No.	Sμ-Sα perfectly matched short homology*	No. of Sμ-Sα fragments analyzed	Switched memory B cells†	Nonswitched memory B cells‡	EUROclass§	Noninfectious complications
6	12.50 ± 2.65‖	11	↓ (0%)	↓ (2%)	smB−	SPM
13	10.70 ± 6.38‖	10	↓ (1%)	↓ (2%)	smB−	AI (ITP) and SPM
4	9.71 ± 7.67‖	8	↓ (0%)	↓ (2%)	smB−	
7	9.17 ± 5.34‖	9	↓ (0%)	↓ (6%)	smB−	
10	8.93 ± 6.33‖	10	↓ (0%)	↓ (2%)	smB−	GR and SPM
25	8.75 ± 6.41‖	8	↓ (1%)	↓ (5%)	smB−	AI (ITP)
15	8.33 ± 6.75‖	10	↓ (0%)	↓ (1%)	smB−	
14	7.57 ± 5.08‖	10	↓ (1%)	↓ (3%)	smB−	
16	7.57 ± 2.15‖	8	↓ (2%)	↓ (2%)	smB−	
11	7.56 ± 3.68‖	9	↓ (3%)	Norm (9%)	smB+	
9	7.10 ± 4.48‖	11	↓ (1%)	↓ (5%)	smB−	
21	7.00 ± 3.92‖	8	↓ (1%)	↓ (5%)	smB−	GR
2	6.60 ± 5.46 (ns)	9	↓ (0%)	↓ (3%)	smB−	
3	6.43 ± 1.99 (ns)	9	↓ (0%)	↓ (2%)	smB−	AI (TID)
1	6.22 ± 4.38 (ns)	9	↓ (0%)	↓ (2%)	smB−	SPM
24	4.89 ± 4.70 (ns)	10	↓ (2%)	↓ (0%)	smB−	AI (ITP)
17	4.71 ± 5.28 (ns)	8	↓ (3%)	↓ (7%)	smB+	
8	4.09 ± 4.37 (ns)	21	↓ (2%)	Norm (15%)	smB−	AI (ITP/AIHA) and SPM
18	3.92 ± 5.53 (ns)	9	↓ (5%)	Norm (14%)	smB+	
23	1.50 ± 1.76 (ns)	7	Norm (10%)	↓ (7%)	smB+	
Control subjects	2.28 ± 3.52	53				
Patients	6.95 ± 5.32¶	194				

† and ‡: ↓, Decreased number; ↑, increased number; *Norm*, within normal range.

AI, Autoimmunity; *AIHA*, autoimmune hemolytic anemia; *GR*, granulomas; *ITP*, idiopathic thrombocytopenic purpura; *ns*, not significant; *SPM*, splenomegaly; *TID*, type I diabetes.

*Means and SDs from patients and control subjects.

†Switched memory B cells = $CD19^+CD27^+IgD^-$ cells.

‡Nonswitched memory B cells = $CD19^+CD27^+IgD^+$ cells.

§smB+ indicates less than 2% of switched memory B cells, and smB− indicates 2% or less switched memory B cells.

‖$P < .0025$, 2-tailed Student *t* test with the Bonferroni correction for multiple comparisons (the same was obtained by applying 1-way ANOVA, followed by the Dunnett multiple comparison posttest with the control treatment as statistical control in Dunnett comparisons).

¶$P < .005$, 2-tailed Student *t* test.

less than −3 SDs with respect to the healthy control group (<18% of the mutated fraction of VκA27) and group B with values ranging between −2 and −3 SDs compared with the healthy control group (≥18% and <28% of the mutated fraction, Table I). Only 1 patient (P25) had values greater than the reference interval of our healthy control group.

We can see that 88% (14/16) of the patients in group A had a low number of switched memory B cells (<2%) belonging to the so-called smB− group of EUROclass (see the Methods section), who also showed clinical complications or more severe presentation of the disease (ie, 6/9 patients with autoimmunity, splenomegaly, or granulomas fell into this group; Table I). On the other hand, 7 of the 8 patients in group B presented with $CD27^+$ B cell counts (total memory B cells) of greater than 5% of peripheral B lymphocytes, and 6 of them have remained thus far free of noninfectious complications (Table I). Interestingly, we found a significant association between patients of group A and the presence of noninfectious complications with respect to patients of group B ($P < .05$, χ^2 test).

In spite of this strong correlation between SHM, the B-cell immunophenotype, and clinical presentation, patient 25 had reference values for the mutated fraction of VκA27, had autoimmunity, and presented switched memory B cells of less than 2% (Fig 1, *B*, and Table I). An important issue is whether the values of hotspot mutations obtained from PBMCs are comparable with those from particular B-cell subsets (ie, memory B cells). For this purpose, we used fluorescence-activated cell sorting to sort $CD27^+$ B cells and performed IgkREHMA analysis from 7 patients (P8, P11, P12, P17, P18, P20, and P23) presenting with more than 2% of switched memory B cells or more than 10% of total memory B cells and also from 4 healthy donors. The analysis showed that these patients, who initially had reduced levels of mutated fractions of VκA27 on total B cells, continued with reduced level of mutations on memory B cells (Fig 1, *D*). Even though the mutation rate could be higher in the switched memory B-cell population with respect to nonswitched memory B cells, even patients with switched memory B-cell counts comparable with those in healthy donors (P20 and P23) had a marked decrease in SHM frequency (Fig 1, *D*). Overall, SHM frequencies ranged to less than the reference interval in patients with CVID, even when the analysis was performed specifically in memory B cells.

Studies of CSR

Patients with CVID demonstrate increased microhomology use. To investigate the quality of the DSB repairs in *in vivo* CSR, we amplified Sμ-Sα regions from genomic DNA of patients and control subjects by using a previously described nested PCR.[21] Despite the low levels of serum IgA in our patients, Sμ-Sα fragments could be amplified, even if with decreased intensity than in healthy donors (data not shown). Therefore, to

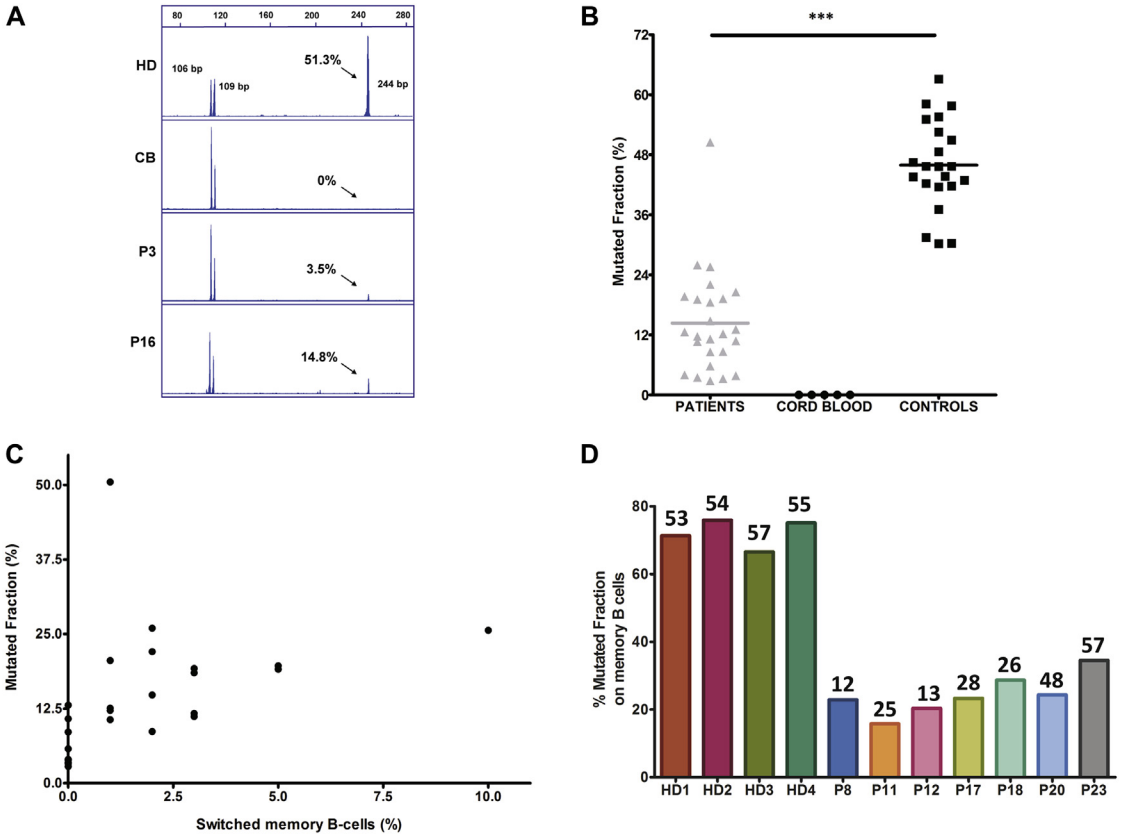


FIG 1. A, IgκREHMA: fragment length analysis using capillary electrophoresis of cut VκA27 PCR products. *CB*, Cord blood; *HD*, healthy donor. Peaks of 106 and 109 bp represent the quantity of VκA27 fragments cleaved in one of the 2 hotspot Fnu4HI restriction sites, respectively. The peaks of 244 bp represent the quantity of VκA27 fragments cleaved in the signal peptide Fnu4HI site but not cleaved in the hot spot as a consequence of 1 or 2 mutations, eliminating the Fnu4HI sites. **B,** SHM values obtained through the IgκREHMA as a percentage of the mutated fraction in patients with CVID *(patients)* and healthy donors *(controls)*. Comparison of means and SDs for the Student 2-tailed *t* test: ***$P < .0001$. **C,** Correlation between the percentage of mutated fraction and the proportion of switched memory B cells in patients with CVID. **D,** Mutated fractions of purified $CD19^+CD27^+$ B cells from 7 patients with CVID *(P)* and 4 healthy donors *(HD)*. Numbers at the top of the bars represent the proportion of switched memory B cells.

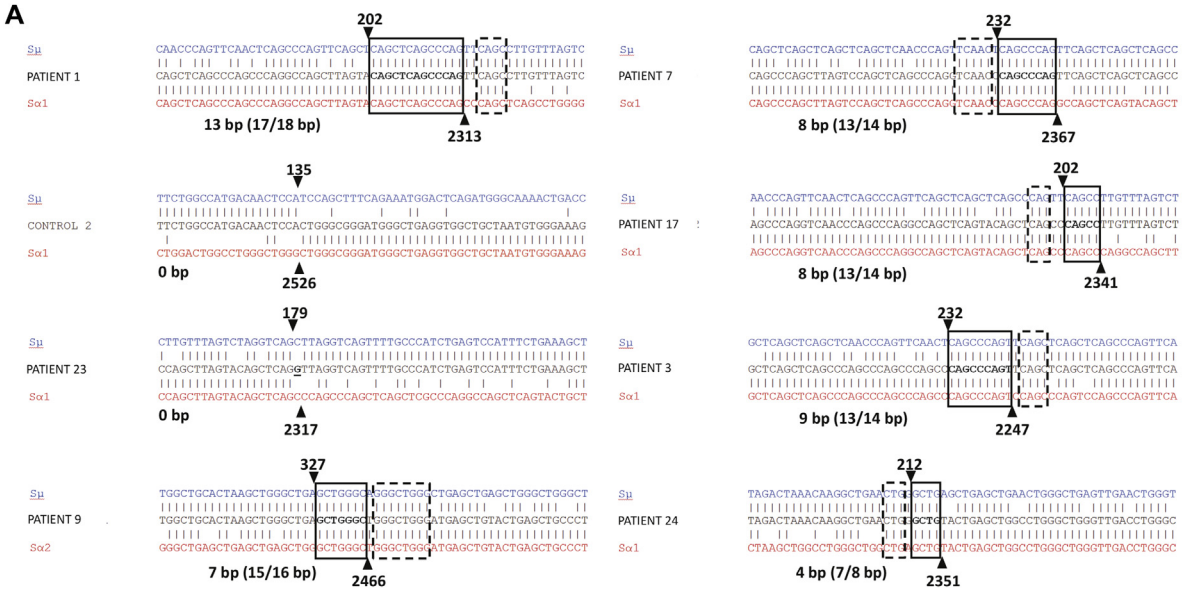
assess the nature of switched junctions, we cloned and sequenced 194 Sμ-Sα fragments from 20 patients with pediatric CVID (Table II) and 53 fragments from 10 healthy control subjects (Fig 2). All of the switched fragment sequences were unique and thus represented independent CSR events. Fig 2, *A*, shows some of the more representative aligned sequences from patients and control subjects.

Globally, we observed a clear decrease in the proportion of blunt junctions (0 bp of homology) in our CVID cohort in comparison with control subjects. Indeed, a significant increase of sequences presenting 7 to 9 bp of microhomology use was observed in patients, even those with more than 10 bp of homology, on the junction repair compared with healthy donors ($P < .001$ and $P < .01$, respectively, χ^2 test; Fig 2, *B*).

Overall, our results showed a significant increase in the extent of donor-acceptor homology on Sμ-Sα joints in patients with CVID with respect to healthy control subjects, with a mean perfect sequence homology of 6.9 ± 5.3 bp in patients with CVID versus 2.3 ± 3.5 bp in healthy control subjects ($P < .0001$, 2-tailed Student *t* test; Fig 2, *B* and *C*). The increase in microhomology use was found in 12 (60%) of the 20 evaluated patients (Table II), and most of the Sμ-Sα joints from these patients were flanked by imperfect repeats (see the dashed line boxes in Fig 2, *A*). Eleven of the 12 patients displaying abnormal microhomology use had less than 2% of switched memory B cells (Table II). The remaining 8 patients had no statistical differences with respect to the control group, and 4 of them presented with more than 10% total memory B cells ($CD19^+CD27^+$, Table II). Despite this increased microhomology use, no significant association with clinical noninfectious complications was observed between abnormal and normal microhomology use subgroups of patients ($P = .26$, χ^2 test).

Altogether, we found a significant correlation between the media of microhomology use in each patient and the proportion of switched memory B cells (Spearman $r = -0.72$, $P = .0003$; Fig 2, *D*), a significant difference that was not noted when compared with IgG or IgA serum levels before substitution with intravenous gammaglobulin (data not shown). These results indicate CSR deficiency in an important group of patients likely associated with a defective repair of switch junctions.

Analysis of TD and TI stimulation further characterizes patient subgroups. In an attempt to localize the precise step of the CSR defect, we tested the ability to generate GLT (IH-CH), CTs (IH-Cμ), and transcripts and to upregulate AICDA in



B

Characterization of Sμ-Sα junctions							
	Perfectly matched short homology						
	0 bp		1-3 bp	4-6 bp	7-9 bp	>10 bp	Total number of S fragments
	No insertions	1 bp insertions					
Controls	16 (30,2%)	10 (18,9%)	15 (28,3%)	4 (7,6%)	5 (9,4%)	3 (5.6%)	53
Patients	**16 (8,2%)*** **	23 (11.9%)	**15 (7,7%)*** **	35 (18,0%)	**55 (28,4%)****	**50 (25,8%)****	194

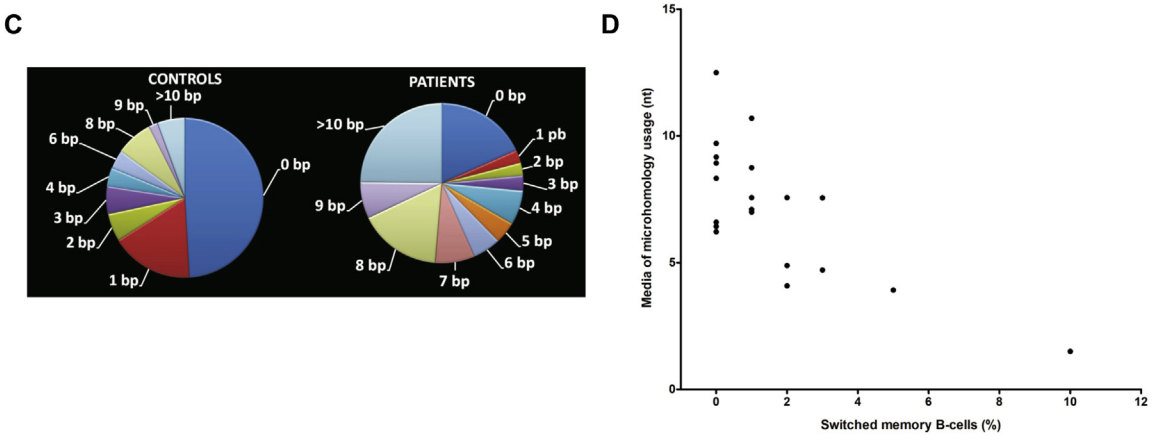


FIG 2. A, Selected sequence of Sμ-Sα junctions from patients and control subjects. Recombination junction sequences are aligned with Sμ *(above)* and Sα or Sα2 *(below)* reference sequences. Microhomology (perfectly matched sequence homology) is indicated by a box *(solid lines)*. The imperfect repeat was determined by identifying the longest overlap region near the switch junction by allowing 1 missmatch on either side of the breakpoints *(dashed line box)*. The Sμ and Sα breakpoints for each switch fragment are indicated as ▲ and ▼, respectively, and their positions in the reference sequences are indicated above or below the *arrowheads*. The number of base pairs involved in microhomology and imperfect repeats for each joint are shown in the bottom of each sequence. **B,** Sμ-Sα switch junctions from patients with CVID were compared with those from healthy donors. Statistical analysis was performed by using the χ^2 test: $^{**}P < .01$ and $^{***}P < .001$. **C,** Pie charts demonstrating microhomology use at Sμ-Sα junctions in patients and control subjects. Proportion of switch junctions with a given size of perfectly matched shot homology is indicated by the size of the slices. **D,** Correlation between the media of microhomology use and the proportion of switched memory B cells.

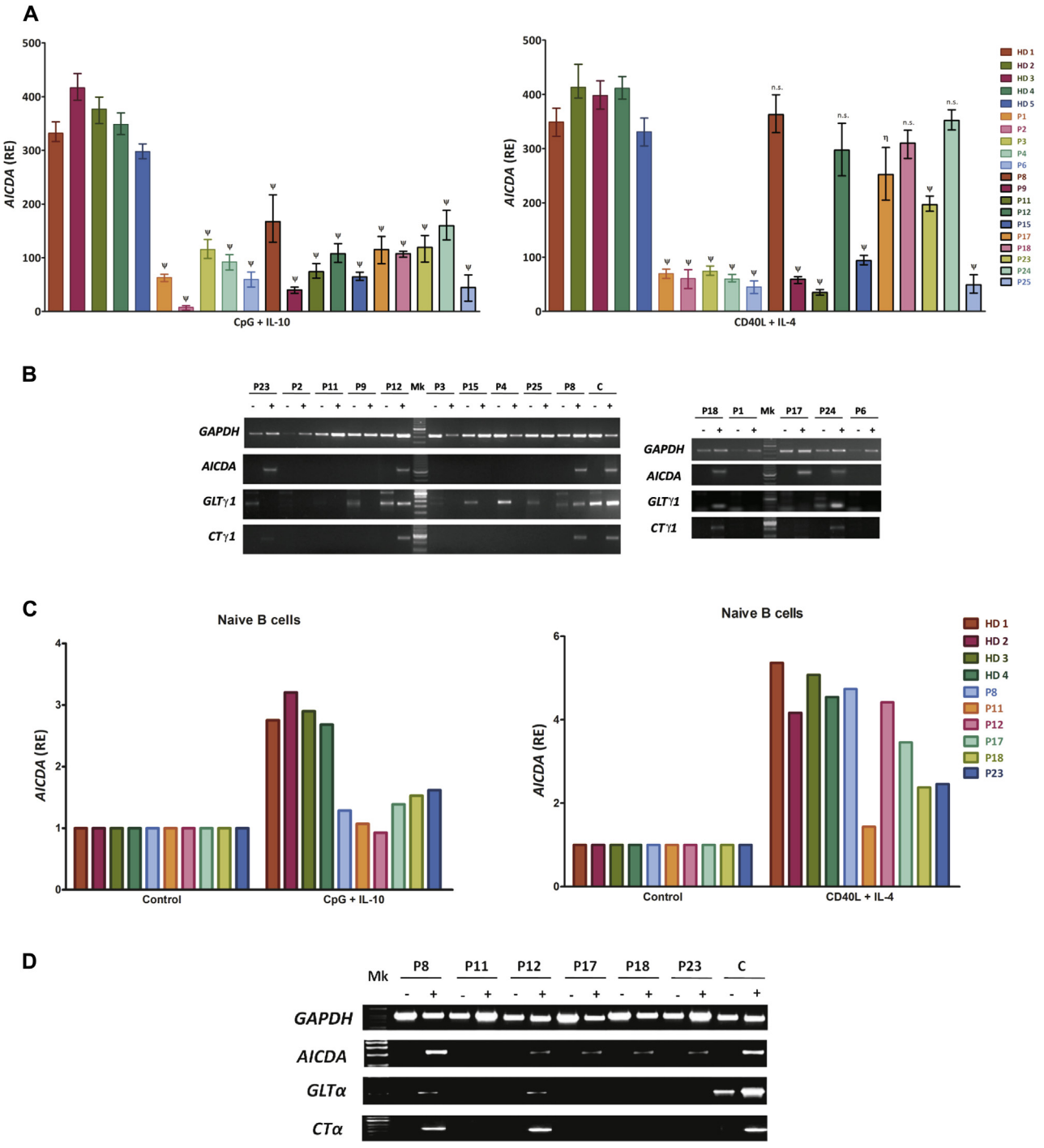


FIG 3. A, qRT-PCR analysis of AICDA in cells of patients and control subjects cultured for 2 or 4 days with anti-CD40 and IL-4 or CpG and IL-10, respectively. *Bars* represent means ± SEMs of 3 independent experiments. Data were analyzed by using the $2^{-\Delta\Delta Ct}$ method with *ACTB* as a reference gene, considering the relative expression *(RE)* of AICDA mRNA from the control culture as 1. Statistical analysis was performed by using 1-way ANOVA, followed by the Dunnett multiple comparison posttest. Ψ*P* < .001; η*P* < .05. *HD*, Healthy donors; *P*, patients. **B,** Expression of AICDA, Iγ-Cγ1 (GLTγ1), Iγ1-Cμ (CTγ1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; charge control) transcripts by using RT-PCR in PBMCs from patients and control subjects before *(−)* and after *(+)* stimulation with CD40L and IL-4. *C*, Control subject; *P*, patient. **C,** qRT-PCR analysis of AICDA in purified naive B cells from 6 patients with CVID and 4 healthy donors cultured for 2 or 4 days with anti-CD40 and IL-4 or CpG and IL-10, respectively. Data were analyzed, as described above. **D,** Expression of AICDA, Iα-Cα (GLTα), Iα-Cμ (CTα), and GAPDH (charge control) transcripts by using RT-PCR in purified naive B cells from 6 patients with CVID and 1 healthy donor before *(−)* and after *(+)* stimulation with CD40L and IL-4. *C*, Control subject; *P*, patient.

PBMCs before and after being activated *in vitro* with TD (CD40L and IL-4) and TI (CpG and IL-10) stimuli in 15 patients (P1, P2, P3, P4, P6, P8, P9, P11, P12, P15, P17, P18, P23, P24, and P25) and 5 healthy donors.

Healthy donors' PBMCs showed an increase in the expression of GLTγ1 (Iγ1-Cγ1) and CTγ1 (Iγ-Cμ; Fig 3, *B*) along with an upregulation of AICDA measured by using RT-PCR and qRT-PCR (Fig 3, *A* and *B*) after TD stimuli (similar results were observed by means of TI stimulation, data not shown). By contrast, none of the patients with CVID showed GLTγ1, CTγ1, and AICDA transcript upregulation by TI stimuli, and they behaved heterogeneously in terms of responsiveness to TD stimuli (Fig 3, *A* and *B*).

Naive B cells obtained from 6 patients with more than 10% of total memory B cells (P8, P11, P12, P17, P18, and P23) and 4 healthy donors were activated *in vitro* to limit possible effects on transcript induction caused by the presence of memory B cells, as previously described (Fig 3, *C* and *D*). We observed similar results through TI stimulation in naive B cells with respect to that observed in PBMCs. However, through TD stimuli, patients 23 and 18 showed less upregulation of AICDA along with a lack of expression of GLTα and CTα transcripts (Fig 3, *D*), as well as GLTγ1 and CTγ1 expression (data not shown).

Taking these results into account, 3 subgroups were delineated: subgroup 1, lacking response to TD stimulation (P1, P2, P3, P4, P6, P9, P11, P15, and P25); subgroup 2, no expressing GLTs but retaining AICDA activation (P17, P18, and P23); and subgroup 3, showing a similar behavior to the healthy control subjects (P8, P12, and P24).

DISCUSSION

In recent years, analysis of several immunoglobulin deficiencies has made it possible to describe molecular mechanisms underlying the CSR and SHM pathways, which are key elements in the maturation of antibody responses.[28] However, functional defects in the ability to perform CSR might or might not be associated with a defect in SHM.[22,29,30] Among the genetic alterations found causing defects on CSR and SHM are those causing hyper-IgM syndromes, such as deficiencies of *CD40* and *CD40L*,[31,32] *AICDA*,[33] or *UNG*[34] genes, which helped us understand the essential role of these molecules in B cells for both mechanisms. Thus the study of the ability of B cells to perform SHM and CSR in patients with primary immunodeficiencies could help to identify new proteins involved either in 1 or both pathways.[7,35-40]

In the present study, by evaluating the qualitative aspects of the antibodies produced by patients with CVID, we described SHM and/or CSR defects in our cohort of pediatric patients. Indeed, even if in our cohort, as reported,[6,7] low or absent switched memory B cells have mainly been associated with secondary complications, this does not seem consistently related to the ability to trigger CSR. Thus intrinsic CSR events remained unaffected in both patients presenting (P12 and P18) and those lacking (P24) switched memory B cells and noninfectious clinical complications. Likewise, by pointing out specific impairments in antibody production, we can attempt to categorize patients with CVID to guide the search for new genetic targets and correlate treatment based on the clinical course of each category.

The results obtained by analyzing the SHM pathway showed that to a greater or lesser extent, almost all patients with CVID had altered SHM of the antibody light chain, making it possible to group them into 2 categories: (1) severe defect in SHM (group A in the IgκREHMA) and (2) moderate defect in SHM (group B in the IgκREHMA, Table I). This assay allows analysis of the frequency of mutated Igk transcripts in a CDR mutation hotspot. It is important to note that mutations in this hotspot seem to be positively selected,[41] and the levels of mutated transcripts correlate highly with the percentage of memory B cells.[12] Indeed, in our cohort heterogeneity of SHM correlated with the percentage of switched memory B cells. This correlation could indicate that the light chain mutation levels might relate on the absence of memory subpopulations; however, as previously observed by Andersen et al,[12] we also found a reduced proportion of the mutated fraction on memory B cells sorted from patients with CVID. Furthermore, even patients with a similar fraction of switched memory B cells as healthy donors (P20 and P23) showed a marked decrease in SHM frequency (Fig 1, *D*). Thus because a low number of memory B cells cannot fully explain the results of the IgkREHMA in our cohort of patients with CVID, an intrinsic defect in the SHM machinery should not be ruled out, at least in some of them.

By analyzing the clinical aspect of patients in each group of altered SHM, we observed that 6 of 9 patients in group A (severe defect in SHM) presented with autoimmunity, splenomegaly, or granulomas (Table I). On the other hand, 6 of the 8 patients in group B remained thus far free of noninfectious complications (Table I). As an important clinical tool, we found a significant association between patients of group A and the presence of noninfectious complications with respect to patients of group B ($P < .05$, χ^2 test). This could potentially make clinicians more aware of noninfectious complications that are more likely to arise in a particular group of patients with CVID and not exhaust valuable resources monitoring patients who are less likely to have noninfectious complications.

To further characterize CVID, B-cell analysis of unique switch junctions representing independent CSR events leads us to distinguish 2 groups of patients: those with a significant increase in the extent of donor-acceptor homology (microhomology) at the Sμ-Sα regions and those with switch junctional sequences statistically comparable with healthy donors (Table II). Despite this increased microhomology use, no significant association with clinical noninfectious complications was observed between abnormal and normal microhomology use subgroups of patients ($P = .26$, χ^2 test).

The mean microhomology use for each patient correlated with the percentage of switched memory B cells (Fig 2, *D*), suggesting that those patients triggering lower but measurable CSR *in vivo* have less donor-acceptor homology on Sμ-Sα joints. By contrast, we could not find any correlation between microhomology use and immunoglobulin serum levels, which seems to be in accordance with the poor predictive value of plasma IgG levels in patients with CVID because even patients with slightly decreased IgG levels can be highly susceptible to infections.[8]

In our attempt to stratify patients with CVID according to SHM or CSR analysis, no difference in infection control became evident. As previously reported, the most frequent clinical manifestation in this cohort of patients with CVID was respiratory tract infection, with 43% presenting with upper respiratory tract infections, namely sinusitis and otitis media, and as many as 79% of patients presenting with recurrent lower respiratory tract infections.[23]

Almost all of the patients in group A according to SHM assay performance presented with failures in CSR-evaluated mechanisms (P4, P6, P7, P9, P10, P11, P13, P14, P15, and P16).

The presence of microhomology and imperfect repeats are reminiscent of defects in the NHEJ repair pathway.[35,42,43] It is important to note that in our CVID cohort no patient presented with clinical or immunologic phenotypes reminiscent of those found in patients with known DNA repair defects. Nevertheless, normal expression of other DNA repair proteins involved in CSR[42,44,45] or in repairing AICDA-induced lesions[38,46] could not be excluded. Similarly, we cannot rule out a defect in a yet uncharacterized molecule involved in NHEJ (S regions in CSR), MMR (Mismatch repair; V regions in SHM), or both or in an alternative pathway of DNA repair involved in both late differentiation processes of B cells. It has been described that patients with mutations in *PMS2* with impaired S-region repair have an altered pattern of SHM.[43] However, this defect has been excluded by using *PMS2* sequencing in our cohort of patients with CVID. The improved DNA-sequencing technologies (ie, next-generation sequencing) should enable us to elucidate the possible gene defects underlying the observed changes in the near future.

In addition, a defect in proteins activated in response to DNA damage and suppressed in B cells through B-cell lymphoma 6 (Bcl-6) should also be considered.[47] Although, to date, no animal model can support this hypothesis, the identification of genes that are directly repressed by Bcl-6 has strengthened the idea of essential and specialized functions of this protein in germinal center formation.[48,49] Bcl-6 appears to be crucial for germinal center B cells undergoing the high rates of proliferation required for CSR and SHM. In fact, it has been shown that Bcl-6 suppresses the responses of cell-cycle arrest and apoptosis through suppression of either p53[50] or p21.[47] In this sense it would also be interesting to evaluate the forkhead box protein O1/AKT/phosphoinositide 3-kinase pathway, which is involved in regulation of apoptosis, cell-cycle arrest, differentiation, and longevity of B cells.[51,52]

On the other hand, patients of group B with moderated defect in SHM but correctly repairing S regions (P17, P18, P23, and P24), a defect in an AICDA cofactor exclusively involved in the activation or repair of the V regions, could be considered. The existence of such a cofactor of AICDA has been previously postulated, but no protein playing that role has been identified.[53] However, although P24 was able to activate CSR molecular events *in vitro* through TD stimuli, P17, P18, and P23 only conserved AICDA activation (Fig 3). Abnormalities involving a first step of CSR, namely GLT activation, could underlie this condition.

Finally, patients 21 and 25 presented with failure in CSR, with a mildly defective SHM evoking the phenotype caused by mutations in the AICDA C-terminal region.[54,55] A cofactor acting at the AICDA C-terminal region, which is specifically required for development of CSR and dispensable for SHM, has been also suggested but not yet identified.[53,56] As an example, defects in the 14-3-3 adapter protein, which is involved in AICDA recruitment to S regions, could be evaluated in these patients,[57] particularly in patient 21, who had an immunologic phenotype reminiscent of defects in genes known to be involved in CSR (ie, hyper-IgM syndromes). However, abnormalities in *AICDA*, *UNG*, *CD40*, *CD40L*, and *PMS2* were ruled out through sequence analysis. Furthermore, normal expression of the inducible costimulator molecule was seen by using flow cytometry, ruling out this deficiency previously described in some patients with CVID.[58,59]

Altogether, molecular definitions of CSR and/or SHM defects in patients with CVID can guide future investigations aiming to further elucidate the complex mechanisms involved in B-cell differentiation pathways.[29,60] Moreover, clinicians could consider assessments of SHM in patients with CVID in view of the observed correlation between severe impairment and noninfectious complications in pediatric patients with CVID.

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Clinical implications: Stratification of our CVID cohort according to their SHM capacity highlighted an inverse relationship with noninfectious clinical complications.

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