CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans

Sandra Weller*, Ahmad Faili*, Corinne Garcia*, Moritz C. Braun*, Françoise Le Deist†, Geneviève de Saint Basile†, Olivier Hermine‡, Alain Fischer†, Claude-Agnès Reynaud*, and Jean-Claude Weill*§

*Institut National de la Santé et de la Recherche Médicale U373, Faculté de Médecine Necker–Enfants Malades, 156, Rue de Vaugirard, 75730 Paris Cedex 15, France; and †Institut National de la Santé et de la Recherche Médicale U429, Hôpital Necker–Enfants Malades, and ‡Unité Mixte de Recherche–Centre National de la Recherche Scientifique 8603 and Département d'Hématologie Clinique, Hôpital Necker–Enfants Malades, 149, Rue de Sèvres, 75743 Paris Cedex 15, France

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Somatically mutated IgM$^+$-only and IgM$^+$IgD$^+$CD27$^+$ B lymphocytes comprise ≈25% of the human peripheral B cell pool. These cells phenotypically resemble class-switched B cells and have therefore been classified as postgerminal center memory B cells. X-linked hyper IgM patients have a genetic defect characterized by a mutation of the *CD40L* gene. These patients, who do not express a functional CD40 ligand, cannot switch Ig isotypes and do not form germinal centers and memory B cells. We report here that an IgM$^+$IgD$^+$CD27$^+$ B cell subset with somatically mutated Ig receptors is generated in these patients, implying that these cells expand and diversify their Ig receptors in the absence of classical cognate T–B collaboration. The presence of this sole subset in the absence of IgM$^+$-only and switched CD27$^+$ memory B cells suggests that it belongs to a separate diversification pathway.

somatic hypermutation | CD27 | X-linked hyper IgM syndrome

In humans, mutated Ig sequences are found exclusively among peripheral B cells in the CD27$^+$ subpopulation (1, 2). This population includes, in addition to classical isotype-switched IgG$^+$ and IgA$^+$ memory B cells, IgM$^+$IgD$^+$ and IgM-only B cells, which comprise respectively about 15% and 10% of the total peripheral B cell population (2–5). Whether these last two subsets correspond to bona fide postgerminal center (GC) cells or whether they could represent a distinct subpopulation of B cells remains an open question (2, 5). It has recently been proposed that a mutated IgM$^+$ population could be generated in mice during a T-dependent immune response, but IgM$^+$IgD$^+$ memory B cells could not be identified formally in this analysis (6). The study of various mammalian B cell immune systems has shown that diversification processes such as hypermutation could be used as an antigen-independent developmental program to generate the preimmune repertoire (7). On the basis of these models, we have looked for the presence of a mutated surface-IgM B cell subset in X-linked hyper IgM (XHIM) patients who harbor a CD40 ligand (CD40L) genetic defect that abolishes CD40-dependent B cell signaling leading to GC formation (reviewed in refs. 8 and 9). Results on XHIM patients have been extremely controversial. At first, Agematsu *et al.* (10) reported the presence of IgM$^+$IgD$^+$CD27$^+$ B cells in XHIM patients but claimed recently that CD27$^+$ memory B cells could not be found in such patients, as anticipated from their lack of GCs (11). Secondly, Ig gene mutations were found in some rare B cells of XHIM patients (12), but this result was put in question in another study in which mutated sequences could only be observed in a case where the CD40L mutation allowed a transient functional expression of the molecule on activated T cells and the generation of IgG$^+$ B cells (13), thus correlating the presence of Ig gene mutations with a leaky CD40L phenotype.

Here we report that patients who have a complete defect in CD40L expression carry a mutated IgM$^+$IgD$^+$ B cell subset in the total absence of IgM$^+$ and switched IgM$^-$IgD$^-$CD27$^+$ B cells.

Materials and Methods

Characterization of the CD40L Mutation in XHIM Patients. cDNAs of XHIM patients were obtained after reverse transcription of the total RNA extracted from peripheral blood mononuclear cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. CD40L transcripts were amplified by PCR and sequenced directly as described (14). In some cases, genomic DNA was also analyzed. The five exons of the *CD40L* gene were amplified with intronic primer pairs (primer sequences available from authors at sbasile@necker.fr), allowing the determination of exon and flanking splice site sequences. PCR products were sequenced directly.

Expression of CD40L on Activated T Cells. Rosette-forming cells (E+) were isolated as described (15), activated with 10^{-8} M PMA (Sigma) and 10^{-6} M ionomycin (Calbiochem) for 4 h, and stained either with (*i*) CD40-Fcγ fusion protein (kindly provided by P. Graber, Serono Pharmaceuticals Research Institute, Geneva, Switzerland) revealed by FITC-conjugated rabbit anti-mouse Fcγ (Jackson ImmunoResearch); (*ii*) mouse anti-human CD40L mAb (IgG1, PharMingen) revealed by FITC-conjugated rabbit anti-mouse Fcγ; or (*iii*) rabbit anti-human CD40L polyclonal Ab (TRAP, kindly provided by R. A. Kroczek, Robert Koch Institute, Berlin) revealed by FITC-conjugated sheep anti-rabbit IgG obtained in our lab. Anti-CD69mAb (Immunotech, Luminy, France) staining was performed in parallel as a control of T cell activation. For each assay, activated control T cells were included. For patients C.Q., C.R., L.P., A.N., and F.F., absence of CD40L expression was also confirmed on activated T blasts established from peripheral blood mononuclear cells cultivated for 6 days in RPMI medium 1640 (GIBCO/BRL) supplemented with 5% human AB serum (BioWhittaker), Con A (5 μg/ml; Sigma), and IL-2 (20 units/ml; Genzyme). Fluorescence analysis was performed on an FACScan Plus (Becton Dickinson).

Separation and Flow Cytometric Analysis of IgM$^+$IgD$^+$CD27$^+$ B Cells. Sorting of M$^+$D$^+$27$^+$ B cells was performed by two-color staining on Ficoll-isopaque–purified cell suspensions enriched in

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Abbreviations: GC, germinal center; XHIM, X-linked hyper IgM; CD40L, CD40 ligand.

§To whom reprint requests should be addressed. E-mail: weill@necker.fr.

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B cells to 95–98% by magnetic cell separation with the MiniMACS system (Miltenyi Biotech, Auburn, CA), and either of the following reagents: (*i*) anti-human IgD-FITC (Caltag, South San Francisco, CA) and biotinylated anti-human CD27 (Ancell, Bayport, MN) plus Streptavidin-TriColor (Caltag); or (*ii*) anti-IgD-FITC, anti-human CD27-PE (Immunotech). This last combination was preferred for the sorting of cells from XHIM patients because the staining of $CD27^+$ populations present at a low percentage was sometimes artificially increased with CD27-TriColor (unpublished observations). The absence of IgD^-CD27^+ memory B cells was monitored on Ficoll-purified peripheral blood mononuclear cells by staining with anti-CD19-PC5 (Immunotech), anti-IgD-FITC, and anti-CD27-PE. Three-color analysis was carried out on gated CD19-PC5–positive B cells. Further characterization was performed on CD19-enriched B cells stained with anti-IgD-FITC, anti-human IgM-PE (Caltag), and biotinylated anti-CD27 followed by Streptavidin-TriColor. Three-color analysis was performed on gated CD27-TriColor–positive cells. Because IgD^+CD27^+ cells coexpress IgM (data not shown), this population is designated as $IgM^+IgD^+CD27^+$ ($M^+D^+27^+$).

Sequence Analysis of Rearranged VH3–23 Gene Segments. Genomic DNA was extracted from sorted D^+27^+ B cells by proteinase K digestion. Rearranged VH3–23 gene segments were amplified from approximately 3,000 cells with Pfu Turbo polymerase (Stratagene) by using a seminested PCR strategy. For the first round of amplification, a VH3–23 leader primer (5′-GGCTGAGCTGGCTTTTTCTTGTGG-3′) and a $3'J_H$ primer mix (5′-TGAGGAGACGGTGACCAGGG-3′ and 5′-TGAGGAGACGGTGACCGTGG-3′ in a 3:1 ratio) were used (45 s at 95°C, 60 s at 64°C, and 90 s at 72°C for 25 cycles). The second round of amplification was performed on 1/10 of the first reaction mixture by using the same 3′JH primer mix and a VH3–23 intronic primer (5′-GTGGAATGGATAAGAGTGA3′) (45 s at 95°C, 60 s at 55°C, and 90 s at 72°C for 25 cycles). The background PCR error value was determined in the same experimental conditions on D^+27^- B cells from cord blood by using the same cell sample size (3,000 cells). Gel-purified PCR products were cloned by using the Zero blunt TOPO PCR cloning kit (Invitrogen). Sequences of VH3–23–positive colonies were performed by using the BigDye cycle sequencing kit (Perkin–Elmer) and analyzed with an ABI310 genetic analyzer. The sequences obtained were compared with the germ-line VH3–23 gene over 288 bp (from Glu-1 to Cys-92).

Results

The IgM^+IgD^+ Subset Is the Only One Present Among $CD27^+$ B Cells in XHIM Patients. We analyzed eight patients who carry a null mutation of the *CD40L* gene (Table 1 and Fig. 1). In none of these patients can CD40L be detected on activated T cells either by monoclonal and/or polyclonal anti-CD40L antibodies or soluble CD40 Fcγ fusion protein. In six patients, a base substitution, an insertion, or a deletion introduces a stop codon, which prevents formation of a complete extracellular tumor necrosis factor-like domain of the CD40L protein (Fig. 1). In the other patients, there is a mutation that abolishes the normal splicing of the molecule (Table 1).

The peripheral B cell population was analyzed according to CD27, IgM, and IgD surface expression in XHIM patients (4–21 yr) and in control samples from cord blood, young children, and adults. XHIM patients lack both $CD27^+$ IgM-only and isotype-switched B cells and only display the $CD27^+IgM^+IgD^+$ subset (Table 2 and Fig. 2). In normal adults, this $M^+D^+27^+$ population varies from 6% to 23% (2). Its size appears to increase with age, from an average of 1% in cord blood to 7% in 4- to 5-year-old children (Table 2). In all but one patient studied, its proportion ranged from 1% to 4%, somewhat lower than in age-matched

Table 1. Invalidating mutations of the *CD40L* gene in XHIM patients

Patients	Serum Ig levels*			Age at diagnosis	CD40L expression on activated T cells			Mutation of *CD40L* gene		
	IgG, g/liter	IgM, g/liter	IgA, g/liter		pAb TRAP	mAb TRAP1	CD40-Fcγ	Genomic DNA mutation†	cDNA mutation	Protein alteration
C.Q.	—	—	—	1 day‡	ND	0§	0	del 10 bp (nt 430–439)		Stop at aa 144¶
C.R.	—	—	—	1 day	ND	0	0	del 10 bp (nt 430–439)		Stop at aa 144¶
L.P.	0.49‖ (2.8–6.8)	0.85 (0.4–0.84)	<0.08 (0.1–0.58)	4 mo	ND	0	0	177G → A	Altered splicing (wild-type mRNA less than 1%)	
A.N.	1.16 (6.8–11.8)	3.96 (0.54–1.14)	<0.08 (0.66–1.34)	4 yr	ND	0	0		Ins 101 bp at nt 367 (L1 element)	Stop at aa 134¶
L.Ch.	0.6 (6.1–10.7)	14 (0.54–1.14)	1.98 (0.46–1)	2 yr	0	0	0	310 (−1)G → T	Del 58 bp (nt 310–367) (exon 3 skipping)	Stop at aa 107¶
Z.A.	0.08 (2.8–6.8)	0.92 (0.4–0.84)	<0.08 (0.1–0.58)	6 mo	0	ND	0		Del 8 bp (nt 432–439)	Stop at aa 138¶
B.M.	0.9 (6.1–10.7)	1.24 (0.54–1.14)	1.1 (0.46–1)	2 yr	0	ND	0	A163 → T		Stop at aa 48
F.F.	0.14 (9.3–14.3)	14 (0.63–1.28)	<0.08 (1–1.94)	11 yr	ND	0	0	C728 → A		Stop at aa 236

aa, amino acid; del, deletion; ins, insertion; ND, not done; p, polyclonal.

*Serum Ig levels at the time of diagnosis before γ-globulin replacement therapy.

†Nucleotide number as described by Hollenbaugh *et al.* (16).

‡Due to an XHIM case in the same family, diagnosis was done immediately after birth for C.Q. and C.R., who are siblings.

§Results are expressed as the % of cells stained by the TRAP antibodies or the CD40-Fcγ fusion protein. Values for positive controls are >50%.

¶Amino acid position in mutant protein.

‖Age-matched control values are indicated in parentheses.

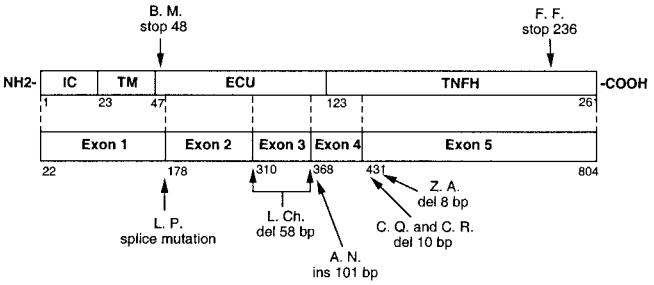

Fig. 1. Location of CD40L mutations in XHIM patients. The schematic representation of the cDNA sequence and of the domains of the CD40L molecule is according to Seyama *et al.* (17). IC, intracellular tail; TM, transmembrane domain; ECU, extracellular unique region; TNFH, tumor necrosis factor-homology domain. For each exon and domain, the starting nucleotide or amino acid residue number is indicated. Nonsense mutations are shown above the scheme, insertions (ins) and deletions (del) below.

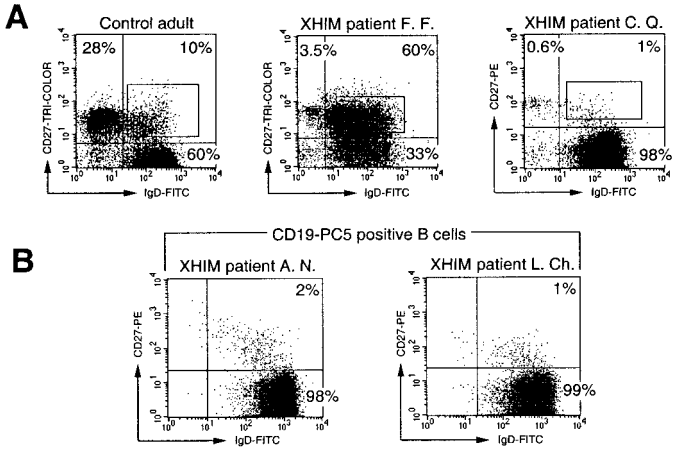

Fig. 2. Fluorescence analysis and sorting of IgD^+CD27^+ peripheral blood B cells of XHIM patients and a control adult donor. (*A*) Anti-IgD/anti-CD27 two-color staining of $CD19^+$ B cells enriched by magnetic cell sorting (MACS) was performed as described in *Materials and Methods*. The gates selected for sorting of the IgD^+CD27^+ B cell population are indicated. The IgD^-CD27^+ population present in XHIM patients corresponds to a residual T cell contamination (see *B* and *Materials and Methods*). The sorted fractions were used for sequence analysis of rearranged VH3–23 gene segments. (*B*) Anti-IgD/anti-CD27/anti-CD19 three-color staining performed on peripheral blood mononuclear cells confirmed the absence of IgD^-CD27^+ memory B cells in XHIM patients. Three-color analysis shows CD27 and IgD expression on $CD19^+$-gated cells. The data are representative of all XHIM patients studied.

controls (Fig. 2 and Table 2). In one case (a 21-year-old patient), there was a striking expansion of the $M^+D^+27^+$ population, well above control adult values (60% of total peripheral B cells). These results emphasize the great variability found in the size of this population, this variability being amplified even in XHIM patients.

The VH3–23 Gene Is Mutated in $M^+D^+27^+$ XHIM B Cells. Somatic mutations were analyzed on rearranged VH3–23 sequences amplified from genomic DNA of sorted $M^+D^+27^+$ B cells. One patient (Z.A.) showed a mutation level close to background, determined in the same experimental conditions on the $M^+D^+27^-$ population. This patient has a specific medical history, since he received (and rejected) a bone marrow graft 3 yr before the present blood sampling. All of the other patients, irrespective of their age, showed a mutation level that resembles the one observed in control children (0.5–1.7% per total sequences and 0.9–1.9% per mutated sequences, with 0–15 mutations per V sequence), except one, who, strikingly enough considering his young age (C.Q., 5 years), showed a mutation frequency closer to a control adult (2.2% per total sequences, 3.27% per mutated sequences, 0–18 mutations per V sequence) (Table 2 and Fig. 3). The overall analysis of sequences showed a normal distribution of mutations with a clustering and a selection for replacement mutations in complementarity determining regions (CDR). In all patients, most of the sequences showed different V_H-D-J_H junctions, indicating the absence of a specific VH3–23 clonal expansion. The variable proportion of unmutated sequences obtained in the $M^+D^+27^+$ population (5–60%) could correspond to the variable purity of the sorted population when present at a low frequency (see *Materials and*

Table 2. Somatic mutations in rearranged VH3-23 genes in $IgD^+IgM^+CD27^+$ peripheral blood B cells of XHIM patients

				Number of sequences		Mutations			
	Donor	Age, yr	% of $D^+M^+27^+$ B cells	Total	Mutated	Range	Number	Frequency/total sequences, %	Frequency/mutated sequences, %
XHIM patients	C.Q.	5	1	28	19 (67%)	0–18	179	2.2	3.27
	C.R.	7	1.5	18	16 (89%)	0–13	90	1.7	1.9
	L.P.	7	2	19	13 (68%)	0–12	61	1.1	1.62
	A.N.	7	2	18	9 (50%)	0–7	27	0.52	1
	L.Ch.	8	1	27	16 (60%)	0–10	41	0.52	0.89
	Z.A.	15	2	24	12 (50%)	0–1	12	0.17	0.34
	B.M.	16	4	23	10 (43%)	0–15	40	0.6	1.33
	F.F.	21	60	20	19 (95%)	0–9	75	1.3	1.37
Healthy controls	D1	4	7	15	8 (54%)	0–14	45	1	1.95
	D2	5	7	19	17 (89%)	0–13	78	1.5	1.7
	D3	16	7	14	13 (93%)	0–19	130	3.22	3.47
	D4	Adult	10	23	19 (83%)	0–22	182	2.75	3.34
Cord blood	C1		1	17	11 (64%)	0–2	12	0.24	0.37
	C2		1	25	5 (20%)	0–2	6	0.08	0.4
	C3		1	16	4 (25%)	0–1	4	0.08	0.34
Background (D^+27^- B cells)				44	12 (27%)	0–2	14	0.1	

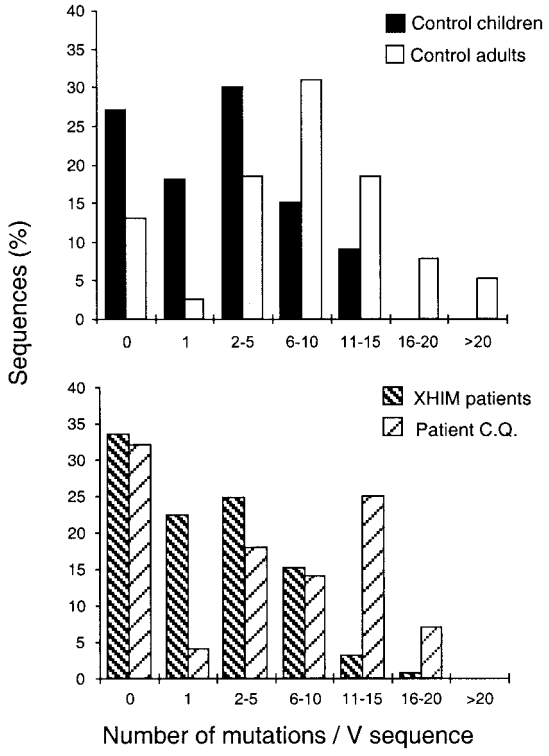

Fig. 3. Distribution of mutations in rearranged VH3–23 gene segments from control donors and XHIM patients. Each histogram represents the percentage of VH3–23 sequences displaying the number of mutations in a given range. Because of his remarkable mutation profile, patient C.Q. was represented separately from the other XHIM patients. (Patient Z.A., for whom mutation frequency was close to background level, was not included in this analysis.) The number of V sequences analyzed in each group is: control children, $n = 33$; control adults, $n = 37$; XHIM patients, $n = 125$; patient C.Q., $n = 28$.

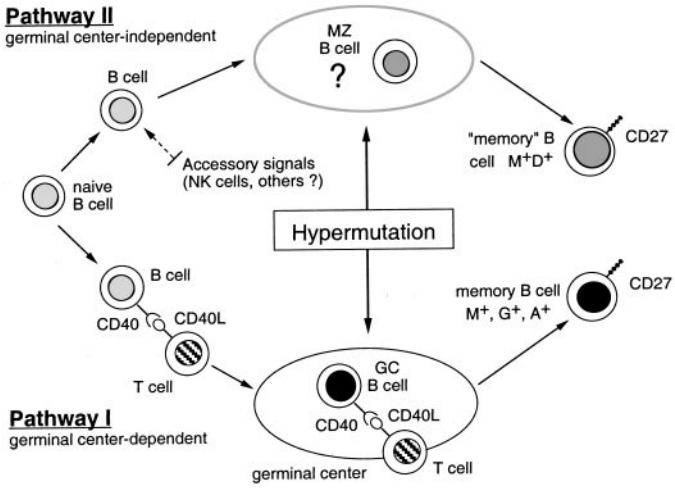

Fig. 4. Proposed scheme of human B cell development leading to Ig gene hypermutation. Pathway I corresponds to T-dependent responses occurring in GCs. Pathway II could correspond to T-independent responses, which may include nonconventional help from natural killer or T cells. The splenic marginal zone (MZ) or equivalent sites in Peyer's patches or lymph nodes could be the site of B cell activation. Ig gene hypermutation takes place in both pathways.

Methods). Accordingly, this proportion was considerably reduced when $M^+D^+27^+$ cells were present in high numbers (e.g., patient F.F. with 60% $M^+D^+27^+$ B cells had 95% mutated V sequences, Table 2). From three control cord blood samples, the mutation frequency in the $M^+D^+27^+$ population was close to background in two cases and slightly above in one case (twice the background level). It is, however, still premature to ascertain whether the mutation process acting in this B cell population may in fact start *in utero*.

Discussion

We report here that XHIM patients with a complete block of CD40L expression harbor a $CD27^+IgM^+IgD^+$ B cell population with mutated Ig genes in the total absence of IgM-only and switched $CD27^+$ B cells. These results confirm the earlier observations by Agematsu *et al.* (10) that XHIM patients display a $M^+D^+27^+$ B cell population. They also explain the results of Chu *et al.* (12), showing that only a small proportion of total B cells in these patients carried mutated Ig sequences because $M^+D^+27^+$ cells represent on average a minor subset of their total B cell population.

The presence of this mutated B cell population allows us to propose the existence of a B cell subset that mutates its antigen receptor in the absence of classical CD40-CD40L mediated T–B interaction leading to GC formation. Although no GCs have been detected either in XHIM patients or in CD40L knockout mice (8, 18, 19), it is not possible to formally exclude that some components of GCs may still be present in these XHIM patients. However, this would imply that because none of them possessed M-only and $M^-D^-27^+$ memory B cells, these residual structures would only allow the formation of memory $M^+D^+27^+$ B cells. This is very unlikely. Accordingly, in mice deficient in lymphotoxin α and lacking organized GCs, repeated immunization lead to the emergence of a bona fide switched memory B cell population with mutated Ig genes (20). CD40 is a pan B cell antigen present on adult and cord blood B cells and also on dendritic cells and macrophages (reviewed in ref. 21). The fact that the $M^+D^+27^+$ subset is not as expanded and diversified in some XHIM patients as in the controls may be explained by the role of CD40L in the regulation of B cell growth and differentiation through the network of cytokine interactions involving dendritic cells, activated T cells, and natural killer cells (9, 22, 23). The latter have been shown to participate in T-independent immune responses (24).

$M^+D^+27^+$ B cells, which represent a major subpopulation in normal individuals (up to 40% of mutated B cells; ref. 2), may therefore be classified outside of the classical post-GC B cell pool, and the same holds true for their malignant counterparts. In fact, it has been found recently, by looking at gene expression profiles with DNA microarrays, that a subgroup of diffuse large B cell lymphoma (DLBCL) carrying a mutated Ig receptor did not display a GC signature and that this new diagnostic marker could allow the definition of distinct clinical entities (25). Our results, which imply that the DLBCL carrying an $M^+D^+27^+$ phenotype may in fact originate from cells belonging to a GC-independent ontogenic pathway, support these conclusions.

In several species belonging to birds and mammals, the preimmune repertoire is generated in gut-associated lymphoid tissues by hypermutation and/or gene conversion, which diversify the three CDRs of rearranged VH and VL genes (7). These B cells can still increase their affinity to T-dependent antigens in GCs, but they are also able as such to sustain high affinity antibody responses to T-independent antigens. It is tempting to speculate that one of the functions of this highly mutated $M^+D^+27^+$ B cell population, which can secrete IgM antibodies *in vitro* (26, 27), could be to generate fast protective responses in humans to T-independent antigens carried by infectious agents such as bacterial polysaccharidic and viral repetitive surface determinants (28, 29). Strikingly enough, although Ig substitution was started late in his life (12 yr), patient F.F., who displays

the most expanded $M^+D^+27^+$ population, never presented most of the bacterial infections usually observed in Ig-treated hypogammaglobulinemic patients (upper respiratory tract infections and pneumonia). One would then like to find out whether these circulating $M^+D^+27^+$ B cells are related to marginal zone B cells, which display a similar phenotype and have been implicated in such responses (30–34).

Whether $M^+D^+27^+$ B cells mutate along an antigen-independent program of development, as in the sheep model of B cell ontogeny (35), or after antigen encounter or both, and whether they receive nonconventional T cell help (36, 37) cannot be answered at this stage. In any case, this observation suggests a new scheme of human B lymphocyte diversification in which IgM-only and switched $CD27^+$ B cells are bona fide GC-derived memory B cells, whereas $M^+D^+27^+$ B cells may develop and mutate along a separate pathway (Fig. 4).

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