Molecular footprints of a germinal center derivation of human IgM+(IgD+)CD27+ B cells and the dynamics of memory B cell generation

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The origin of IgM+CD27+ B lymphocytes with mutated IgV genes, which account for ~20% of human peripheral blood (PB) B cells, is controversially discussed. A generation in a primary diversification pathway, in T cell–independent immune responses, or in T cell–dependent germinal center (GC) reactions has been proposed. We show here that IgM+IgD+CD27+ and IgM+IgD-/lowCD27+ B cell subsets carry, like class-switched memory B cells, mutations in the *Bcl6* gene as a genetic trait of a GC experience. Moreover, the identification of PB IgM+IgD+CD27+ B cells clonally related to GC-derived IgG+ memory B cells with shared and distinct IgV gene mutations demonstrates the GC origin also of the former subset. These findings provide genetic evidence for a GC derivation of somatically mutated IgM+ B cells and indicate that adult humans harbor a large population of IgM+IgD+ post-GC memory B cells. Furthermore, the analysis revealed that a highly diverse and often very large population of memory B cells is generated from a given GC B cell clone, and that (preferentially IgM) memory B cells are generated already early in the GC reaction. This provides novel insights into the dynamics of GC reactions and the generation of a memory B cell repertoire.

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Abbreviations used: BCR, B cell receptor; CDR, complementarity determining region; FR, framework region; GC, germinal center; MMC, major mutation cluster; PB, peripheral blood; SHM, somatic hypermutation; SNP, single-nucleotide polymorphism; TD, T cell dependent; TI, T cell independent.

Upon activation by T cell–dependent (TD) antigens, naive B cells are recruited into germinal centers (GCs; MacLennan et al., 1997). With appropriate stimulation by helper T cells and dendritic cells, the GC B cells clonally expand, diversify their Ig variable (IgV) region genes by somatic hypermutation (SHM), and are selected for increased affinity of their B cell receptor (BCR; Rajewsky, 1996). Independent from the process of SHM, class switching of the Ig heavy chain constant region from IgM to downstream Ig isotypes occurs in GC B cells (Shan et al., 1990; Harriman et al., 1993). Accordingly, somatically mutated IgV genes in combination with the expression of a class-switched BCR were considered as the hallmarks of the descendents of GC B cells, i.e., memory B cells and post-GC plasma cells (Hayakawa et al., 1987; Weiss and Rajewsky, 1990).

Approximately 10 yr ago, two IgM+ B cell subsets with mutated V genes were described in humans; they are a population of cells characterized by high expression of IgM but low IgD surface levels (IgM-only B cells) and IgM+IgD+CD27+ B cells (Klein et al., 1997, 1998; Tangye et al., 1998). Nearly all cells of these subsets carry somatically mutated IgV genes, but their mean mutation load is ~50–60% of that of class-switched memory B cells. As both mutated IgM+ B cell populations and the class-switched memory B cells share expression of the TNF-receptor superfamily member CD27, whereas unmutated naive B cells lack its expression, this marker was proposed to represent a general human memory B cell marker (Agematsu et al., 1997; Klein et al., 1998). The idea that the two IgM+ B cell subsets with mutated V genes are GC-derived memory B cells was supported by the observations that these cells are rare in newborn infants but accumulate with age (Agematsu et al., 1997; Zandvoort et al., 2001), are long-lived (Richards et al., 2000), show a propensity like class-switched memory B cells to differentiate into Ig-secreting

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cells upon appropriate stimulation (Agematsu et al., 1997; Bernasconi et al., 2002, 2003; Shi et al., 2003), and respond in vitro to various stimuli more similar to class-switched memory cells than to naive B cells (Tangye et al., 2003a, b; Good et al., 2006; Good and Tangye, 2007).

However, recent studies on human $IgM^+IgD^+CD27^+$ B cells indicated that other mechanisms might be responsible for the generation and maintenance of these cells. Based on a reported dependency of these cells on the splenic environment, which is known to be important for T cell–independent (TI) type II immune responses, it was proposed that $IgM^+IgD^+CD27^+$ (as well as IgM-only B cells, which were not distinguished in that work) are generated in TI responses (Kruetzmann et al., 2003). In another scenario, it was proposed that the mutated $IgM^+IgD^+CD27^+$ B cells underwent SHM in a primary, antigen-independent diversification mechanism outside GC, as it is known to occur in sheep and rabbits (Weinstein et al., 1994; Reynaud et al., 1995). This idea was based on the observation that x-linked hyper-IgM patients apparently lack GC (caused by defective CD40L-signaling) and class-switched, as well as IgM-only, B cells, but still have substantial numbers of mutated $IgM^+IgD^+CD27^+$ B cells (Weller et al., 2001, 2004). Several findings supported this scenario, including an analysis of CDRIII length diversity of the respective subsets, particularly the presence of rare mutated IgM^+CD27^+ B cells in fetal liver (Scheeren et al., 2008) and neonatal cord blood (Weller et al., 2001), as well as the detection of transcripts of activation-induced cytidine deaminase, an enzyme that is essential for SHM and class switching, in human cord blood transitional type 1/immature B cells (Kuraoka et al., 2009).

As $IgM^+IgD^+CD27^+$ B cells and IgM-only B cells account for ~15 and 5% of human peripheral blood B cells, respectively, and occur at similar frequencies also in secondary lymphoid tissues (Pascual et al., 1994; Dunn-Walters et al., 1995), the cellular origin and functions of about a quarter of human mature B cells are currently controversially discussed.

To clarify whether mutated IgM^+ B cells are post-GC cells, we analyzed molecular footprints of GC reactions in human B cell subsets. It has been shown that Bcl6, the master transcriptional regulator in GC B cells (Klein and Dalla-Favera, 2008), is affected by the SHM machinery in GC B cells, resulting in ~30% of GC and class-switched memory B cells carrying *Bcl6* mutations in the intronic major mutation cluster (MMC; Pasqualucci et al., 1998; Shen et al., 1998; Peng et al., 1999). As SHM of IgV and non-Ig genes is strictly dependent on high-level transcription of the target sequences (Fukita et al., 1998; Bachl et al., 2001; Yang et al., 2006), *Bcl6* should only be mutated in GC B cells that express high levels of this gene, but not in non-GC B cells that lack *Bcl6* expression or show only low transcript levels. Thus, if the vast majority of IgM^+CD27^+ B cells acquired somatic IgV gene mutations in a GC-independent way, and hence in the absence of *Bcl6* transcription, these cells should lack *Bcl6* mutations. Second, if $IgM^+IgD^+CD27^+$ and class-switched B cells derive from common GC B cell clones, one may find clonally related $IgM^+IgD^+CD27^+$ and class-switched B cells in the PB, and these cells should carry shared and distinct somatic V gene mutations.

RESULTS

Bcl6 mutations in human $CD27^+$ B cell subsets

We analyzed the mutation status of the *Bcl6*-MMC in FACS-sorted B cells from three healthy adult blood donors by single-cell PCR. This approach allows the reliable detection of rare mutations, as PCR-introduced mutations are negligible in the direct sequence analysis of the amplificates from single cells. As it is relevant for the determination of the mutation frequency whether one or both alleles of a cell were amplified, we analyzed donors who were prescreened to carry a known single-nucleotide polymorphism (SNP; rs3832246, 1 bp deletion) on one allele of *Bcl6*, so that for unmutated sequences it could also be determined whether one or both alleles were amplified. Overall, from ~21% of the cells (range 14–25%) both alleles were amplified (Table I). The *Bcl6*-MMC of all 60 PCR-positive naive B cells was unmutated, whereas 16 of 75 (21%) $IgM^+IgD^+CD27^+$ B cells, 13 of 64 (20%) IgM-only, and 19 of 57 (33%) class-switched B cells carried mutations. In the $CD27^+$ B cell subpopulations, 8–12 cells had a single mutation and 4–9 cells harbored 2–6 mutations per sequence. The mean mutation frequencies were 0.045% (range 0.036–0.084%) for $IgM^+IgD^+CD27^+$ B cells, 0.050% (range 0.013–0.119%) for IgM-only B cells, and 0.085% (range 0.082–0.093%) for class-switched B cells. Thus, $IgM^+IgD^+CD27^+$ and IgM-only B cells carry class-switched B cells in a fraction of cells *Bcl6* mutations as a footprint of a GC passage of these B cells. The mutations were distributed throughout the whole MMC, and besides two 1-bp deletions and one 5-bp deletion, the mutations were single basepair substitutions with a bias for transitions in $IgM^+IgD^+CD27^+$, IgM-only, and class-switched B cells (63, 58, and 46%, respectively, as compared with an expected frequency of 33% for unbiased mutations). The overrepresentation of transitions over transversions was statistically significant only for both IgM^+ B cell subsets ($P < 0.001$ each), but not in those of class-switched B cells ($P < 0.09$). The RGYW and WRCY motifs are known SHM hotspots (Rogozin and Kolchanov, 1992), and each of the three $CD27^+$ B cell subsets had an enrichment of mutations in RGYW/WRCY motifs. 28.6% of the mutations in $IgM^+IgD^+CD27^+$, 18.5% in IgM-only, and 40.5% in class-switched B cells were within the motifs, in comparison to an expected frequency of 16.7%, assuming unbiased targeting of these motifs. The difference between observed and randomly expected targeting of RGYW/WRCY motifs reached statistical significance only for class-switched B cells ($P < 0.001$). However, in another study *Bcl6* mutations in class-switched B cells from human tonsil also did not show significant RGYW targeting (Yavuz et al., 2002). The fact that the intrinsic SHM biases analyzed did not reach statistical significance in all instances might be caused by the restricted number of mutation events analyzed and/or by the fact that some *Bcl6* mutations influence *Bcl6*

Table I. *Bcl6* mutations in human PB B cells

Donor	Cell type	PCR efficiency[a]	1 allele/2 alleles amplified[b]	Mutated cells	Number of mutations[c]	Mutation frequency[d]
				(%)		%
A	naive	26/50	22/4	0	0	0
	$IgM^+IgD^+CD27^+$	31/50	22/9	7 (23)	6×1, 1×2	0.039
	IgM-only	26/50	21/5	5 (19)	5×1	0.024
	class switched	18/50	16/2	6 (33)	4×1, 1×2, 1×5	0.082
B	naive	18/50	11/7	0	0	0
	$IgM^+IgD^+CD27^+$	25/50	21/4	5 (20)	4×1, 1×3	0.036
	IgM-only	20/50	15/5	6 (30)	1×1, 2×2, 1×4, 1×5, 1×6	0.119
	class switched	24/50	19/5	8 (33)	4×1, 2×2, 1×3, 1×5	0.082
C	naive	16/40	12/4	0	0	0
	$IgM^+IgD^+CD27^+$	19/40	15/4	4 (21)	2×1, 1×5, 1×6	0.084
	IgM-only	18/40	13/5	2 (11)	2×1	0.013
	class switched	15/40	14/1	5 (33)	2×1, 2×2, 1×4	0.093
mean	naive	60/140	45/15	0	0	0
	$IgM^+IgD^+CD27^+$	75/140	58/17	16 (21)	12×1, 1×2, 1×3, 1×5, 1×6	0.045
	IgM-only	64/140	49/15	13 (20)	8×1, 2×2, 1×4, 1×5, 1×6	0.050
	class switched	57/140	49/8	19 (33)	10×1, 5×2, 1×3, 1×4, 2×5	0.085

[a]Numbers of positive PCRs versus single cells analyzed.
[b]Number of PCR products containing one/two alleles.
[c]In A × B, A denotes the number of sequences with B mutations.
[d]The percentage of mutations in a total of 670 bp of the *Bcl6*-MMC considered from each sequence.

expression (Pasqualucci et al., 2003) and hence may be selected for or against.

Clonal relation of human $IgM^+IgD^+CD27^+$ and class-switched memory B cells

If IgM^+ memory B cells and class-switched B cells both are descendants of common GC B cell clones, it should principally be possible to find clonally related IgM^+ and IgG^+ B cells with shared and unique mutations in PB. We focused on $IgM^+IgD^+CD27^+$ B cells because their origin is more controversial than that of IgM-only B cells (Weller et al., 2001), and for our analysis it would have been difficult to obtain enough cells from the small IgM-only subset.

Our first approach to identify such clonally related cells was based on a PAGE strategy and aimed at very large clones. Considering the number of cells that could be used per PCR reaction (30,000) and the total number of IgG^+CD27^+ and $IgM^+IgD^+CD27^+$ B cells in the PB ($\sim 6 \times 10^8$ per subset), we calculated that a putative clone should have at least 20,000 members in each of the two subsets to be detectable by this approach. Several aliquots of 30,000 IgG^+CD27^+ and $IgM^+IgD^+CD27^+$ B cells each were sorted from PB of 10 healthy donors. Extracted RNA was separately reverse transcribed with gene-specific primers for Cγ and Cμ/Cδ and amplified with a V_H1 family-specific primer and the same Cγ and Cμ/Cδ primers (Fig. 1 A). The Cγ primer used in this and the experiment described further below is specific for the Cγ1 and Cγ3 genes, which are classically used by post-GC memory B cells, but does not amplify Cγ2 transcripts, which mainly derive from TI responses (Shackelford et al., 1988). All C_H primers were complementary to positions in equal distance from the first coding nucleotide of the corresponding C_H gene. Thus, an identical CDRIII length of putative clone members in $IgM^+IgD^+CD27^+$ and IgG^+CD27^+ B cells would result in equal-size products in a CDRIII-spectratyping analysis, performed on neighboring lanes of a PAGE gel. Such bands of interest were cut out from the gel, reamplified, and sequenced. Overall, 120 pairs of identical length were analyzed from sorted cells of the 10 donors. Most of these products (85%) were oligo- to polyclonal and not further followed up. From the monoclonal products in one instance, clonally related *V_H1-18*–expressing IgM and IgG transcripts were obtained, and after cloning of the amplificates 7 distinct IgG^+ and 2 distinct IgM^+ members of the clone were identified (Fig. 2 A). The clone members contained between 3 and 9 V_H gene mutations per sequence, of which 1 to 7 were shared by at least two clone members. Importantly, the genealogical tree depicting the most likely clonal evolution revealed that several IgG^+ clone members descended from a proposed intermediate with only a single point mutation,

whereas the two IgM sequences shared 5 additional point mutations with the other IgG^+ sequences. We conclude that the IgM^+ B cells acquired most if not all of their mutations in the clonal evolution of the GC B cell clone that gave rise to these memory B cells.

As the gel-based approach was restricted by the relatively small number of cells that could be analyzed for a clonal relationship, we established an RT-PCR–based approach with clone-specific primers to screen larger cell samples (Fig. 1 B). RNA from 10^6 to 1.5×10^6 sorted IgG^+ and $IgM^+IgD^+CD27^+$ B cells isolated from 8 adult peripheral blood donors, and in two instances from naive B cells (donors 7 and 8), was extracted and reverse transcribed with Cγ and Cμ/Cδ primers, respectively. The cDNAs from both subsets were amplified with primers for V_H6-FR1 (donors 1–6) or V_H3-*23* leader peptide sequence (donors 7 and 8) in combination with nested constant region primers. The nesting effect of the second constant primer set prevents the amplification of $V_HD_HJ_H$-rearrangements from potential cell sorting contaminations, i.e., IgM transcripts from an IgG^+ B cell sort and vice versa. By analyzing the single member V_H6 family, we aimed to enhance the chance to find clonally related cells caused by a reduced heterogeneity of the population. However, as V_H3-*23* is one of the most frequently used V_H gene segments (Brezinschek et al., 1995), we aimed to clarify whether clonally related IgM^+IgD^+ and IgG^+ B cells can also be found for such a gene segment. The amplified V_H6 and V_H3-*23* rearrangements of IgG^+ B cells were cloned and a number of sequences (8 to 30 per PB donor) were randomly chosen to design CDRIII-specific primers. These primers were mainly binding to the 3′ end of CDRIII (and in several instances parts of FRIV), so that enough sequence from the 5′ part of CDRIII was included in the resulting amplificates to allow an unequivocal determination of the clonal relationship of the amplified rearrangements. The CDRIII primers were used together with the V_H6 or V_H3-*23* primer to amplify clonally related sequences from the V_H6-Cμ/Cδ– or V_H3-*23*-Cμ/Cδ–amplified cDNA from $IgM^+IgD^+CD27^+$ B cells, respectively.

From donors 1–6, we were able to amplify and directly sequence 21 monoclonal PCR products from V_H6-expressing $IgM^+IgD^+CD27^+$ B cells (range 2–5 per donor; oligo- or polyclonal amplificates caused by cross-hybridization of the "clone-specific" primers on other V_H6 gene rearrangements were not further considered). Of these 21 products, 11 V_H6

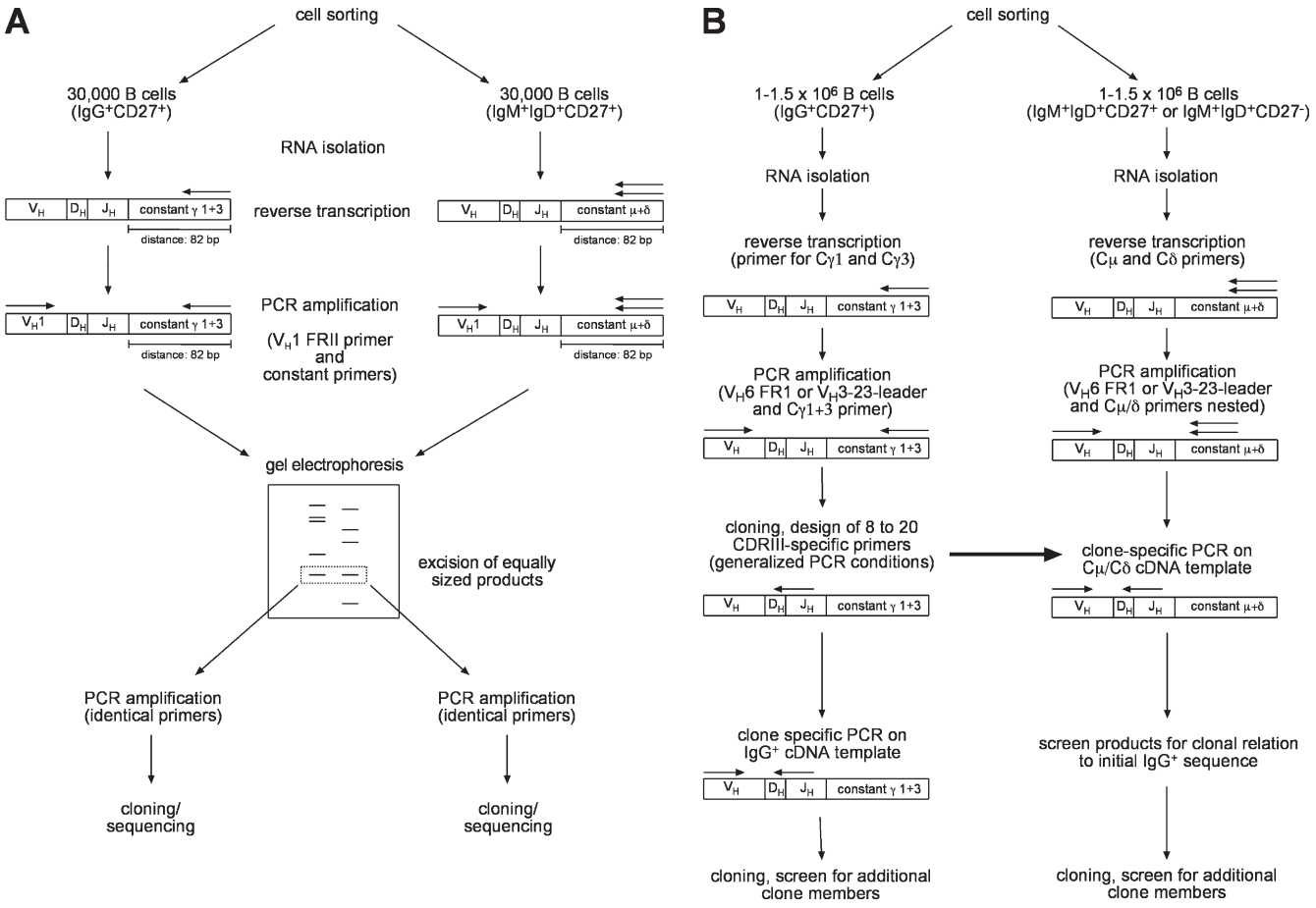

Figure 1. Flow charts of the experimental approaches used to identify clonally related sequences in PB IgM^+ and IgG^+ B cells. (A) PAGE–based approach. (B) CDRIII-specific primer–based approach.

rearrangements from $IgM^+IgD^+CD27^+$ B cells were clonally related to the respective V_H6 rearrangements from the IgG^+ B cells (Table II). From donors 7 and 8, we obtained 9 and 7 monoclonal PCR products, respectively, from *V_H3-23*–expressing $IgM^+IgD^+CD27^+$ B cells. Of these 16 products, 3 *V_H3-23* rearrangements from $IgM^+IgD^+CD27^+$ B cells were clonally related to the respective *V_H3-23* rearrangements from the IgG^+ B cells (Table II). With the "clone-specific" primers for these three memory B cell clones, no clonally related sequences were obtained from the corresponding naive B cells, although 14–21 sequences from the cloned PCR amplificates were analyzed for the three clones. Instead, the sequences from the naive B cells consisted of 4–10 different unmutated sequences with varying CDRIII length and composition (Fig. S1). As one would not expect to find naive B cell clone members among the isolated cells, the lack of CDRIII sequences shared by the naive and IgG^+ B cells validates the reliability of the approach to identify clonally related sequences among the mutated IgD^+IgM^+ and IgG^+ B cells.

To study the intraclonal diversity of the clonally related V_H-Cμ and V_H-Cγ amplificates, for each of the 11 *V_H6* and 3 *V_H3-23* clones, PCR products obtained with the CDRIII-specific primers from the IgG^+ and $IgM^+IgD^+CD27^+$ B cells were cloned into plasmids and several of these were sequenced. Sequence alignments of five selected sequences per clone, including the initial IgG sequence, are depicted in Fig. S2.

For all 14 clones expressing *V_H6* or *V_H3-23*, multiple distinct members, differing from each other by single, few, or >24 point mutations were found (Fig. 2). The overall mutation frequency of the IgG^+CD27^+ B cells (including clone A from the PAGE analysis) is 6.2% and of the $IgM^+IgD^+CD27^+$ B cells 4.2%. Genealogical trees were generated to visualize the mutational evolution of the clones, and a heterogenous picture was obtained (Fig. 2). Moreover, in 6 clones, IgM members are mainly early progeny of the clones and hence carry relatively few mutations (Fig. 2, clones H-M), whereas the IgG^+ post-GC B cells – with few exceptions – have acquired more shared and distinct mutations. In the other 9 clones, including clone A (Fig. 2, A–G, N, and O), IgG^+ and IgM^+ clone members are found more intermingled. Notably, also in these trees, IgG sequences on average carry more mutations than IgM sequences. Unmutated IgM^+ sequences were detected in two clones (Fig. 2, E and L), and unmutated IgM and IgG sequences were detected in one clone (Fig. 2 G).

Considering the overall shapes of the trees depicting the clonal evolution of the B cell clones, a striking diversity is evident. Each clone consists of a high number of branches, and many distinct sequences were found. This shows that throughout the GC reaction, and starting early during clonal expansion and mutation accumulation, clone members are selected to leave the GC and become members of the memory B cell pool.

Assuming that all clone members in a sample were detected (certainly an underestimation), we estimated the total number of clone members in the PB of the donors. Counting repeatedly identified sequences only once, we calculated a clone size of 190 B cells for the smallest clone (Fig. 2) and 3,600 B cells for the largest clone (Fig. 2 M) detected in our study (see Materials and methods).

DISCUSSION

Human IgM-only and $IgM^+IgD^+CD27^+$ B cell subsets carry molecular footprints of a GC reaction

To clarify the still unresolved origin of somatically mutated IgM^+ B cells in humans, we analyzed mutations in the *Bcl6* gene as a genetic trait of a previous GC experience. These mutations are found in ∼30% of GC B cells and class-switched memory B cells and show key features of SHM (Pasqualucci et al., 1998; Shen et al., 1998). As the mutation frequency in *Bcl6* is ∼50–100-fold lower than the one of IgV genes (Pasqualucci et al., 1998), this explains why such mutations are not found in all class-switched memory B cells. Importantly, SHM is strictly dependent on transcription of its target genes (Fukita et al., 1998; Bachl et al., 2001; Yang et al., 2006). On this basis, we reasoned that *Bcl6* mutations should only be present in IgM^+ B cells with mutated IgV genes when these cells acquire their mutations in a GC reaction, i.e., when *Bcl6* is strongly transcribed (Klein and Dalla-Favera, 2008). Indeed, although there is a low basal level of *Bcl6* transcription in mature resting naive human B cells (Ye et al., 1993), this is further reduced when the cells undergo TI immune responses outside GC (Allman et al., 1996; Toellner, K.-M., personal communication). Thus, *Bcl6* should not acquire mutations in case SHM would be active in TI responses. We cannot formally exclude that Bcl-6 is transcribed during the proposed primary V gene diversification pathway for the generation of somatically mutated $IgM^+IgD^+CD27^+$ B cells; however, this seems very unlikely considering that Bcl6 is the master regulator of the GC B cell differentiation program (Klein and Dalla-Favera, 2008).

As somatic mutations in IgV genes and *Bcl6* are generated by the same SHM machinery (Pasqualucci et al., 1998), one would expect that their levels are correlated, and this is exactly what we observed. Both IgM^+CD27^+ B cell subsets have ∼50–60% of the mutation load of class-switched memory B cells in their IgV genes and in *Bcl6*. In conclusion, the detection of *Bcl6* mutations in $IgM^+IgD^+CD27^+$ and IgM-only B cells strongly argues that most if not all of these cells derive from a GC reaction.

Derivation of $IgM^+IgD^+CD27^+$ and IgG^+CD27^+ B cells from common GC B cell clones

If one assumes that $IgM^+IgD^+CD27^+$ and IgG^+CD27^+ B cells derive from common GC B cell clones, and hence both represent post-GC memory B cells, finding of clonally related IgM^+ and IgG^+ PB B cells would represent an important validation for this assumption. However, a clonal relationship as such does not discriminate between the three proposed origins of somatically mutated $IgM^+IgD^+CD27^+$ B cells. If these cells acquire IgV gene mutations during an

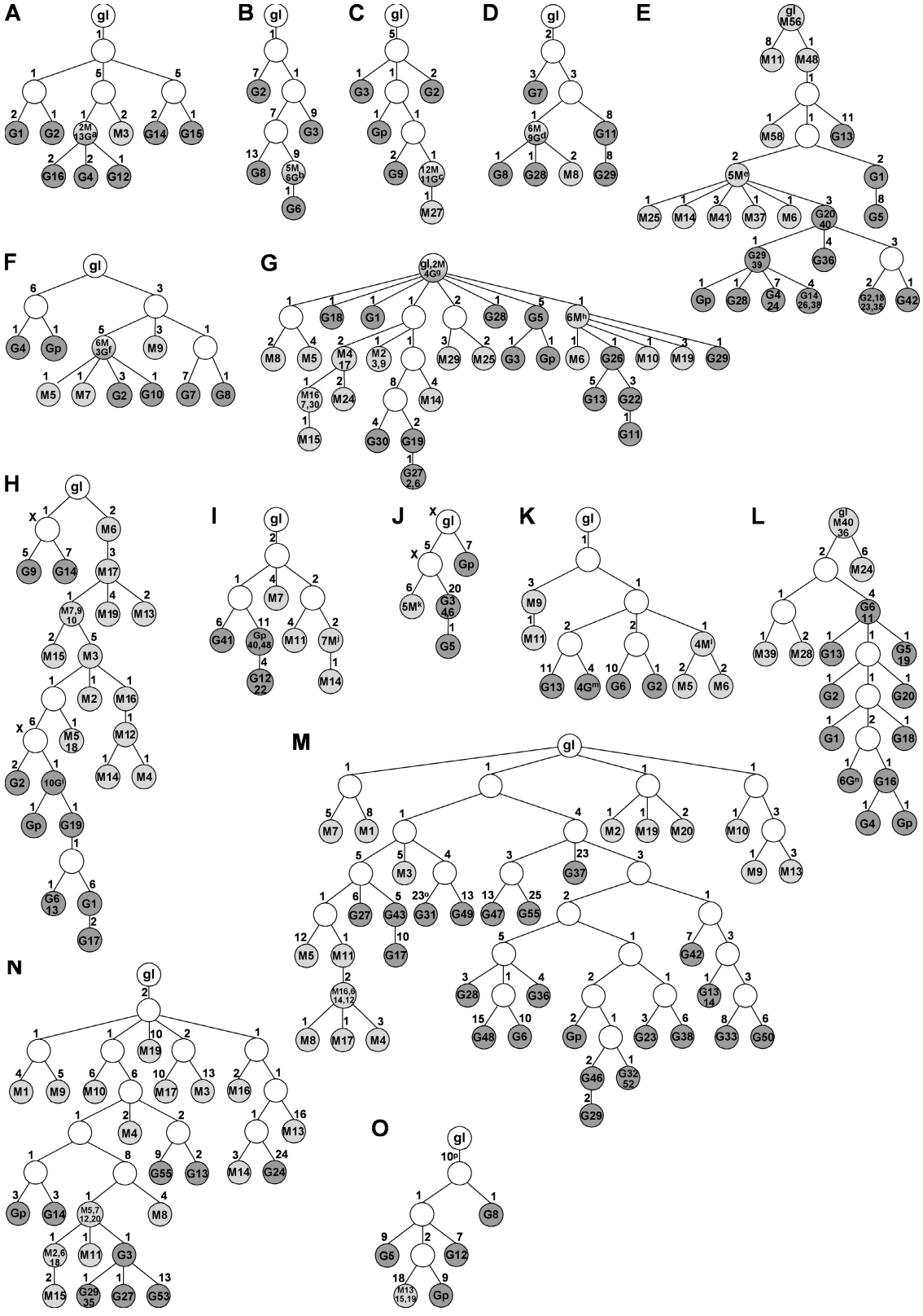

Figure 2. Genealogic trees of *V_H1-18*–, *V_H6*–, or *V_H3-23*–expressing memory B cell clones, reconstructed from clonally related IgM$^+$ and IgG$^+$ sequences. Abbreviations in the circles represent single sequences from IgM$^+$ (M) or IgG$^+$ (G) B cells, empty circles represent hypothetical intermediates. Gp denotes the IgG$^+$ B cell sequence that was used to design the clone-specific primer. Numbers besides the lines indicate the number of (additional) somatic mutations acquired from a given cell to its descendent. Each tree is rooted by the putative, unmutated V_H rearrangement, marked as "gl" (the

Table II. Clones with IgM+ and IgG+ members in human PB memory B cells

Donor	V_H-gene analyzed	CD27+ B cells	CDRIII primers[a]	Products with monoclonal CDRIII[b]	Clones with IgM and IgG members[c]
		%		(%)	(%)
1	V_H6	30	15	5 (33)	2 (40)
2	V_H6	40	20	2 (10)	0
3	V_H6	45	20	3 (15)	0
4	V_H6	30	15	3 (20)	3 (100)
5	V_H6	25	8	4 (38)	2 (50)
6	V_H6	19	8	4 (50)	4 (100)
7	V_H3-23	29	30	9 (30)	1 (11)
8	V_H3-23	21	30	7 (23)	2 (29)

[a]Number of CDRIII-primers which amplified a specific product from diluted DNA of the respective IgG+ B cell clone.
[b]Counted are only those products which gave rise to a sequence with distinct CDRIII, i.e. those where the CDRIII-primer specified a unique clone.
[c]The number of clones with IgM+ and IgG+ B cells. The frequency of clones among monoclonal products is shown in brackets.

antigen-independent primary diversification process, these cells may also enter GC reactions upon TD activation and give rise to IgG+ memory B cells. Even if IgM+IgD+CD27+ B cells represent GC independent memory B cells from TI immune responses, such cells may, in particular circumstances, be driven into GC reactions and become IgG+ memory B cells. It is only the mutation pattern within a clone that can discriminate between the different scenarios. Several of the clones are indeed unhelpful in clarifying this issue. In three clones shown in Fig. 2 (I, K, and L), all IgG+ clone members may potentially derive from one prediversified IgM+ B cell or a memory B cell from a TI response that was driven into a GC reaction. However, in two other clones (Fig. 2 H) such a scenario is already less likely, as here one would have to postulate that two different members of a prediversified clone were driven into GC reactions. Importantly, the pattern of mutation accumulation in the other 10 clones (Fig. 2, A–G and M–O) is incompatible with a non-GC dependent scenario. In these clones, IgG and IgM members are intermingled in the genealogical trees, and some of the IgG cells can be found at earlier branches in the trees than IgM members, despite a number of shared mutations. Thus, in these clones IgG+ and IgM+ members coevolved from a common GC B cell clone.

The selective search for clonally related IgG+CD27+ and IgM+IgD+CD27+ B cells does not allow one to determine whether all or only a small fraction of IgM+IgD+CD27+ B cells were generated in GC reactions. Nevertheless, the fact that we found clonally related IgM+IgD+CD27+ B cells for ∼40% of the informative IgG+ memory B cells (in total, 14 of 37 Cγ transcript sequences tested with monoclonal Cμ or Cδ amplificates) indicates that GC B cell clones regularly give rise to both IgM and IgG memory B cells. That we did not find IgM+ members for all clones may simply be caused by technical matters (e.g., mutations in IgM+ clone members at primer binding sites) or a smaller size of many memory B cell clones, so that in the aliquot of PB that we analyzed no IgM members were present (note that we sampled ∼10% of PB B cells, but as only 2% of lymphocytes are present in PB [Blum and Pabst, 2007], overall we analyzed only an aliquot of 0.2% of B cells). Moreover, it is remarkable that in many of the clones the number of (distinct) members was similar for IgM+ and IgG+ B cells, which indicates that for at least half of the IgG+ memory B cells a population of clonally related IgM+ memory B cells of similar size exists. Considering that the frequency of IgG+ memory B cells in the PB is similar to the frequency of IgM+CD27+ B cells, this, together with the results of the *Bcl6* mutation analysis, suggests that indeed most if not all of the IgM+CD27+ B cells in human adults are post-GC B cells.

How do these findings relate to the indications for a generation of somatic mutations in IgM+IgD+CD27+ (and IgM-only) B cells by a prediversification mechanism or in the course of TI immune responses? Regarding an origin of IgM

roots of clones E, G, and L also include unmutated members). The trees were constructed to maximize the number of shared and minimize the number of unique mutations. In rare instances, a reversion of a formerly shared mutation was assumed to ensure consistency of the dendrogram. Importantly, the overall shape of the respective tree was never affected. In two instances (clones H and M), the development of IgG clones members could principally be explained by two independent prediversified IgM clone members (marked with an "x") entering GC reactions. (A) Includes M1, M2, G6-13, and G17-21. (B) Includes M1, M2, M6, M8, M10, G1, G4, G5, G7, G9, and Gp. (C) Includes M2, M7, M14, M19, M20, M24, M28, M31, M33, M37, M39, M40, G1, G5, G7, G8, G10, G17, G21-24, and G29. (D) Includes M2-6, M9, G4, G9, G18, G19, G21-23, G27, and Gp. (E) Includes M15, M28, M43, M49, and M63. (F) Includes M1-4, M6, M10, G1, G3, and G9. (G) Includes M12, M21, G4, G7, G8, and G10. (H) Includes M13, M20, M23, and M26-28. (I) Includes G3-5, G7, G8, G10-12, G15, and G18. (J) Includes M1-3, M8, M10, M12, and M15. (K) Includes M3, M4, M6, M9, and M10. (L) Includes M2, M8, M10, and M15. (M) Includes G10, G11, G14, and Gp. (N) Includes G3, G9, G10, G14, G15, and G17. (O) The number of mutations includes a 3-bp deletion in sequence G31. The number of mutations includes a 3-bp insertion in all downstream sequences.

memory B cells from TI responses, it may well be that a small fraction of these cells were indeed generated in such immune reactions. However, the reported specific dependence of IgM memory B cells from the spleen with its marginal zone, and hence the association with TI type II responses (Kruetzmann et al., 2003), was not seen in several other studies (Weller et al., 2004; Wasserstrom et al., 2008; Martinez-Gamboa et al., 2009), and there is evidence from studies in rodents that the marginal zone is also a reservoir for post-GC memory B cells (Liu et al., 1988; Song and Cerny, 2003), so a relationship between PB IgM^+CD27^+ B cells and splenic marginal zone B cells does not argue against a GC derivation of the cells. Regarding the evidence for a prediversification mechanism in $IgM^+IgD^+CD27^+$ B cells, the recent description of a small population (3–5% of IgD^+ B cells) of somatically mutated $IgM^+IgD^+CD27^+$ B cells in fetal tissues indeed argues for a GC-independent development of these cells (Scheeren et al., 2008). Importantly, however, only ~20% of these fetal IgM^+CD27^+ B cells harbor somatic mutations, and the mutation load in these mutated cells was only about half of that of $IgM^+IgD^+CD27^+$ B cells in adults (Klein et al., 1998). Another main argument for a GC-independent origin of mutated $IgM^+IgD^+CD27^+$ B cells relates to their presence in children affected by the x-linked hyper IgM syndrome (Weller et al., 2001). However, in these patients only a fraction of $IgM^+IgD^+CD27^+$ B cells carried mutated V genes, and a recent study indicated that the mutation frequency of the mutated V genes is often considerably lower than that of the corresponding B cells from healthy individuals (Longo et al., 2009). Moreover, in x-linked hyper IgM patients, there could be an unphysiological expansion of the prediversified $IgM^+IgD^+CD27^+$ B cells in the absence of competition with a normal population of memory B cells for survival niches. Finally, it may also be that GC-like structures exist in such patients and that memory B cells generated in such structures preferentially give rise to $IgM^+IgD^+CD27^+$ B cells in the absence of CD40-triggering for class-switching. Taking these considerations together, a picture is emerging in which some $IgM^+IgD^+CD27^+$ B cells are initially generated during early development in a primary antigen-independent diversification process, with a relatively low mutation load. Later in life, the majority of cells with this phenotype appear to derive from GC reactions and hence represent post-GC memory B cells.

Dynamics of memory B cell generation in the GC

The detection and characterization of rearranged IgV genes from memory B cell clones not only showed that IgG^+ and $IgM^+IgD^+CD27^+$ memory B cells can derive from the same GC B cell clone, but provided additional insights into the dynamics of memory B cell generation in the human. First, GC B cell clones give rise to memory B cells throughout the GC reaction, and begin to do so very early in the response, as evident from PB memory B cells with very few or even a lack of IgV gene mutations. This finding provides evidence that lowly mutated memory B cells are not derived from particular immune reactions associated with diminished V gene diversification and/or hampered T cell help. Moreover, the detection of unmutated members of memory B cell clones might indicate that the few unmutated B cells that were described in previous V gene studies of $CD27^+$ B cells are (mostly) memory B cells and not cell sorting contaminants. Also class switching is initiated very early during the clonal expansion, as multiple IgG^+ clone members with few somatic mutations were detected. Second, the population of memory B cells generated from a GC B cell clone is often very diverse in terms of V gene sequences. This implies that memory B cells with a wide spectrum of affinities and antigen-binding characteristics is generated from a GC B cell clone. This might facilitate the recognition of slightly modified pathogens in recall responses by the memory B cell pool. Third, although IgM and IgG cells are often found intermingled in the genealogical trees, there is a clear tendency that IgM^+ memory B cells were generally generated earlier than IgG^+ memory B cells and hence show a lower overall mutation load (4.2% for IgM^+ cells versus 6.2% for IgG^+ memory B cells). These values are the same as those determined previously for the populations of $IgM^+IgD^+CD27^+$ and IgG^+ memory B cells (Klein et al., 1998). Therefore, our analysis of clonally related $IgM^+IgD^+CD27^+$ and IgG^+ memory B cells indicates that a main determinant for the lower mutation load of the mutated IgM^+ B cells is their earlier generation during GC reactions, and not, for example, a generation in separate, perhaps less efficient GC responses.

Concluding remarks

The results of the present study indicate that there is a large population of IgM^+ memory B cells in human adults. Although the lower mutation load of these cells as compared with class-switched memory B cells may imply that the antibodies produced by these cells are usually of lower affinity that the one of IgG antibodies, this may be compensated for by the higher avidity of the pentameric IgM complexes when B cells differentiate into plasma cells and secrete antibody. Moreover, these memory B cells may serve as a flexible memory B cell reservoir that may reenter GC upon encounter with the same or a similar antigen to undergo further affinity maturation and be able to switch the isotype of their BCR to the needs of the particular recall response.

Besides the importance of revealing the origin of about a quarter of the human B cell pool, the present work also has important implications regarding the origin and consequence of dysregulated B cells in autoimmune diseases and immunodeficiencies, as well as B lymphoid malignancies. For example, many mature B cell lymphomas with somatically mutated IgV genes express IgM (and often also IgD), and the present study therefore supports the view that these are GC B cell–derived lymphomas (Küppers et al., 1999).

MATERIALS AND METHODS

Cell separation. Buffy coats of healthy adult donors were obtained from the blood bank of the Institut für Transfusionsmedizin of the Medical School

of the University of Duisburg-Essen. Donors for full blood donations were recruited from the Medical School. All samples were collected with informed consent of the donors. The study protocol was approved by the Internal Review Board in Essen. PB mononuclear cells were isolated by Ficoll-Paque density centrifugation, and $CD19^+$ B cells were enriched to >98% by magnetic cell separation using the MACS system (Miltenyi Biotec).

Cell sorting. The B cell–enriched cell suspensions were stained with CD27-APC (eBioscience), anti–human IgD-PE, and IgM-FITC or IgG-FITC antibodies (all from BD). B cell subpopulations were sorted with a FACSDiva cell sorter (BD) as naive B cells ($IgM^+IgD^+CD27^-$), class-switched memory B cells (IgG^+CD27^+), $IgM^+IgD^+CD27^+$ B cells ($IgM^+IgD^+CD27^+$), and IgM-only B cells ($IgM^+IgD^{-/low}CD27^+$). Purity was >99% for each population as calculated by reanalysis on a FACSCanto flow cytometer (BD) in combination with FACSDiva software.

***Bcl6* mutation analysis.** Single cells were sorted into Expand High Fidelity PCR buffer (Roche) and incubated with 0.25 mg/ml proteinase K for 2 h. A semi-nested PCR strategy was used to amplify the *Bcl6*-MMC with a length of 744 bp (Pasqualucci et al., 1998). The first round of amplification was performed in 2.5 mM $MgCl_2$, 125 μM dNTPs, 0.125 μM each primer (5′-CGCTCTTGCCAAATGCTTTGGC-3′ and 5′-CTCTCGTTAGGAAGATCACGGC-3′), and 1.2 U High Fidelity DNA polymerase mix (Roche). For the second round of amplification, 1.5 μl of the first round reaction were used as template. PCR conditions were 1.75 mM $MgCl_2$, 67 μM dNTPs, 0.125 μM each primer (5′-CGCTCTTGCCAAATGCTTTG-3′ and 5′-GACACGATACTTCATCTCATC-3′), and 1.2 U Fermentas Taq DNA polymerase. For sequence analysis, PCR products were purified with EZNA Cycle pure kit (VWR International GmbH) and sequenced from both strands with second round amplification primers. The procedure was accomplished using the ABI Cycle Sequencing Kit, version 3.1 on an ABI3130 Sequencer (Applied Biosystems). Sequence analysis was performed using the SeqScape software version 2.5 (Applied Biosystems) and the GenBank data library. To calculate whether one or two alleles were amplified from a cell, donors were prescreened for heterozygosity of a known monoallelic SNP in the *Bcl6*-MMC, a 1-bp deletion (rs3832246).

Clonal relation in PB B cells: PAGE strategy. IgG^+CD27^+ B cells and $IgM^+IgD^+CD27^+$ B cells were sorted (as described in the Cell sorting section) in 30,000 cell aliquots. RNA was extracted with RNeasy micro kit (QIAGEN) and RT was performed with Superscript-III-RT (Invitrogen) according to the manufacturer's protocol. For gene-specific RT of RNA from class-switched B cells, a primer was used that recognizes Cγ1 and Cγ3 (5′-AGGCAGCCCAGGGCCGCTGTGCC-3′), and for RNA from $IgM^+IgD^+CD27^+$ B cells a mixture of a Cμ (5′-AGCCAACGGCCACGCTGCTCGTAT-3′) and a Cδ primer (5′-CATGCCAGGACCACAGGGCTGTTATC-3′) was used. The distance to the 3′ end of the rearranged J_H gene is identical for the three primers. V_H1 family-specific cDNA was amplified in 1.5 mM $MgCl_2$, 250 μM dNTPs, 0.125 μM each primer (VH1FR2 5′-GGTGCGACAGGCCCCTGGACAA-3′ and constant primers as in RT), and 1.2 U High Fidelity DNA polymerase mix (Roche) in 35 cycles. PCR products were precipitated, resolved in 10 μl formamide and heat-denatured at 95°C for 2 min. Samples were loaded in neighboring lanes of a 6% denaturing PAGE gel (19:1 acrylamide/bisacrylamide, 7 M urea in TBE buffer). The gel was stained with the Silver sequence DNA staining kit (Promega) according to the manufacturer's instructions. PCR products of equal size in both lanes were excised and watered O/N in 50 μl H_2O. 5 μl were reamplified in additional 35 PCR cycles and sequenced with the VH1FR2 primer. Products with identical, monoclonal CDRIII in both populations were cloned using pGEM-T-Easy cloning kit (Promega) and sequenced to identify clone members (Fig. 1 A).

Clonal relation in PB B cells: CDRIII-primer strategy. From 1 to 1.5 × 10^6 cell aliquots of IgG^+CD27^+ B cells and $IgM^+IgD^+CD27^+$ B cells from donors 1–8 and of naive B cells from donors 7 and 8, cDNA was prepared as described in the previous section and amplified in 1.5 mM $MgCl_2$, 250 μM dNTPs, 0.125 μM each primer (V_H6FR1 5′-GGTGCGACAGGCCCCTGGACAA-3′ or V_H3-*23*-leader peptide sequence 5′-TTTGGGCTGAGCTGGCTTTTTCTTGTG-3′ and either a Cγ RT-primer or nested primers for Cμ 5′-TCGTATCCGACGGGGAATTCTCACAG-3′ and Cδ 5′-GTTATCCTTTGGGTGTCTGCACCCTG-3′), and 1.2 U High Fidelity DNA polymerase mix (Roche) for 35 cycles. The cDNA of IgG^+ B cells was cloned using the pGEM-T-Easy cloning kit (Promega), and multiple plasmids per cloning were sequenced. For 8–30 sequences of each donor, CDRIII-specific primers were generated. PCR conditions were determined that were suitable to amplify for most of the clone-specific primers products from the corresponding diluted plasmid preparations (5 ng/μl) in no more than 35 cycles. PCR for the detection of clonally related sequences from cDNA of $IgM^+IgD^+CD27^+$ B cells and naive B cells from donors 7 and 8 was performed with the following conditions: 30 μl reaction mixture containing 1.5 mM $MgCl_2$, 250 μM dNTPs, 0.125 μM each primer (V_H6FR1 or *VH3-23* leader peptide primer and CDRIII-specific primer), primary denaturation step of 95°C for 4 min, followed by 35 cycles of 30 s at 95°C, 30 s at 62°C, 60 s at 72°C, and 5 min at 72°C. Resulting amplificates were cloned and multiple plasmids were sequenced. CDRIII-specific primers that amplified clonally related sequences in IgM^+ B cells were in parallel used to amplify further clone members from IgG^+ cDNA (see flowchart in Fig. 1 B). All distinct V_H gene sequences are available at Genbank/EMBL/DDBJ under accession nos. FN562934–FN562983, FN564030, and FN563492–FN563831.

Mutation tree analysis. Sequences with identical CDRIII were aligned by Clustal W (Megalign; DNAStar). The number of expected DNA polymerase errors per sequence was determined using the following formula: ∼200 bp sequenced × 70 PCR cycles × polymerase error rate (8.3×10^{-6}/bp/cycle [Roche]) = 0.1. Neglecting the low number of 1 expected polymerase induced error per 10 sequences, all nucleotides that did not match germline configuration of the respective V_H gene were considered as mutations introduced by SHM. To create the IgV gene lineage trees, the sequences were manually positioned in genealogic order, rooted by the germline configuration and assuming that a high number of shared mutations in a sequence determines a late ancestor and vice versa. Each set of sequences was analyzed in parallel with the IgTree program (Barak et al., 2008; provided by R. Mehr, Bar-Ilan University, Ramat Gan Israel). Each genealogic tree was constructed to obtain a minimal amount of branching points and mutation events. In case the particular sequence of single mutation events could not be determined (e.g., possible back mutations were not outweighed by shared mutations or shared mutations could as well be interpreted as independent events), the solution in favor of a higher number of shared mutations is shown. Importantly, the basic structure of the tree was not affected.

Calculation of clonal sizes. Assuming all members of a clone in the sorted cell aliquots were detected by the PCR-based strategy and counting repeatedly identified sequences as one, we calculated the potential clone size in the PB. For example, for the largest clone (Fig. 2 M, donor 7) for which we detected 37 members in the 3×10^6 sorted $CD27^+$ B cells, in the total PB with $\sim 1 \times 10^9$ $CD19^+$ B cells (10^8 B cells in 500 ml PB, total blood volume of ∼5 liter), of which 29% were $CD27^+$, this clone would have in 5 liter of PB a size of ∼3,600 B cells (37×10^9 x 0.29: 3×10^6). The sizes of the other clones were analogously determined.

Online supplemental material. Fig. S1 shows the V_H sequences obtained from the naive B cells of donors 7 and 8 using V_H3-23– and CDRIII-specific primers for clones M, N, and O. Fig. S2 shows five selected sequences of each clone (A-P), aligned to the germline configuration of the corresponding V-, D-, and J-segments. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091087/DC1.

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