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Evidence for preferential Ig gene usage and differential TdT and exonuclease activities in human naïve and memory B cells

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Abstract

Memory B cells and the antibodies they encode are important for protective immunity against infectious pathogens. Characterization of naïve and memory B cell antibody repertoires will elucidate the molecular basis for the generation of antibody diversity in human B cells and the optimization of antibody structures that bind microbial antigens. In this study we aimed to investigate the influence of antigenic selection on the antibody genes of the two CD27⁺ memory B cell subsets, comparing them with the naïve repertoire in CD27⁻ cells. We analyzed and compared the Ig heavy chain gene transcripts in three recently defined circulating naïve and memory B cell subsets (CD19⁺IgD⁺CD27⁻ [naïve], CD19⁺IgD⁺CD27⁺ [un-class-switched memory] or CD19⁺IgD⁻CD27⁺ [class-switched memory]) at the single cell level. We found similar biased patterns of variable, diversity and joining heavy chain gene usages in all three groups of cells. CD19⁺IgD⁺CD27⁺ memory B cells harbored as diverse an antibody gene repertoire as CD19⁺IgD⁻CD27⁺ memory B cells. Interestingly, CD19⁺IgD⁺CD27⁺ memory B cells possessed a lower frequency of somatic mutations, a higher incidence of exonuclease activity at the 3' end of D regions, and a lower frequency of N and P nucleotide additions at both V_H-D and D-J_H junctions of CDR3 regions compared to CD19⁺IgD⁻CD27⁺ memory B cells. These data suggest distinct functional mechanisms underlying selection of this unique subset of un-class switched memory B cells.

Keywords

Human; B cells; Antibodies; Memory; Repertoire development

Introduction

The generation of highly diverse antibodies with high affinity is a key element of acquired immunity (Bernasconi et al., 2002; Crotty and Ahmed, 2004). However, the molecular and

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cellular basis for development of diverse and effective antibody repertoires remains only partially elucidated. Classically, memory B cells were considered B cells that had class-switched from the initial expression of surface IgD and IgM to that of other Ig classes, resulting in surface expression of IgG, IgA, or IgE, with a lack of IgD. Recent progress in B cell phenotyping has clarified that an isotype-switched phenotype identifies only a subset population of memory B cells. On the basis of recent research, adult circulating B cells can be separated on the basis of memory markers and isotypes into at least three subpopulations: 1) IgD⁺CD27⁻ naïve B cells, 2) IgD⁺CD27⁺ un-class-switched memory B cells, and 3) IgD⁻CD27⁺ class-switched memory B cells (Agematsu, 2000; Agematsu et al., 2000; Klein et al., 1998; Shi et al., 2003).

The molecule CD27, a type I glycoprotein expressed on memory B cells and the majority of T cells, is a member of the tumor necrosis factor receptor family (Camerini et al., 1991; Prasad et al., 1997). Accumulating data about CD27 indicate that this molecule is present on the surface of memory B cells but not naïve B cells, and CD27 signaling promotes the differentiation of memory B cells into plasma cells (Agematsu, 2000; Agematsu et al., 2000; Klein et al., 1998; Nagumo et al., 1998; Raman et al., 2003; Shi et al., 2003). IgD⁺CD27⁺ un-class-switched memory B cells in peripheral blood have been shown to be a distinct subpopulation of memory B cells that may play a crucial role in secondary immune responses because of their prompt synthesis of high-affinity IgM molecules (Shi et al., 2003). *In vivo*, the origin and function of IgD⁺CD27⁺ B cells are still uncertain. It has been demonstrated that IgD⁺ B cells residing in human tonsils can acquire somatic mutations in variable genes without switching isotypes (Liu et al., 1996). Hyper-IgM (HIGM) patients who carry an invalidating mutation of the *CD40L* gene do not possess normally developed germinal centers or switched memory B cells but still have a subpopulation of circulating IgD⁺CD27⁺ B cells, suggesting that the IgD⁺CD27⁺ B cells might form a B cell subset distinct from classical germinal center-derived memory B cells (Weller et al., 2001). A recent study suggested that IgD⁺CD27⁺ B cells correspond to circulating splenic marginal zone B cells, based on phenotypic analysis, complementarity determining region 3 (CDR3) spectra-typing and gene-expression profiling of blood and splenic B cell subsets (Weller et al., 2004). Analysis of this peripheral subset of B cells in healthy children younger than 2 years further indicated that these B cells could develop and mutate their Ig receptor during ontogeny even before a functional splenic marginal zone matures.

To date, detailed molecular characterization of antibody genes expressed in these naïve and memory B cell subsets is limited. Klein *et al.* reported mutational analysis of 67 rearranged V_H genes isolated from IgD⁺CD27⁺ memory B cells and 32 rearranged V_H genes from IgD⁺CD27⁻ naïve B cells by using genomic PCR specific for only 3 of the 7 V_H gene families, V_H 1, 3, and 4 (Klein et al., 1998). In that study, the mutation frequency of IgD⁺CD27⁺ memory B cells was 3.7%, 5.0%, and 5.9% respectively for 3 healthy adult donors. In a more recent report, Weller *et al.* studied the mutation frequency of one V_H gene, V_H3-23, in the two memory B cell groups and showed a lower mutation frequency of B cells (3.8% versus 5.7% in IgD⁻CD27⁺ memory the V_H3-23 gene in IgD⁺CD27⁺ memory B cells) (Weller et al., 2004). Detailed characterization of naïve and memory B cell antibody gene repertoires will facilitate better understanding of molecular mechanisms underlying the regulation of memory B cells responses, and the generation and expansion of antibody diversity.

In this study, we simultaneously isolated single cells from the three subsets of human circulating naïve and memory B cells from healthy adult volunteers based on the surface expression of IgD and CD27. Using single B cell culture and multiplex reverse transcription PCR designed to amplify all Ig variable region gene segments in the VBASE complete database of genomic variable gene sequences, individual Ig variable region genes of Ig heavy chains from single cells were cloned and analyzed. We found a similar pattern of biased Ig gene usages

among the three subsets of naïve and memory B cells, suggesting a highly conserved biased antibody repertoire in human memory B cells despite previous antigen exposure. Our data confirmed the differences in mutation frequencies in the two memory B cell groups. Furthermore, characteristics in the CDR3 regions observed in the IgD⁺CD27⁺ memory B cells compared with IgD⁻CD27⁺ memory B cells suggested differential levels of terminal deoxynucleotidyl transferase (TdT) and exonuclease activities during the generation of the two subsets of memory B cells.

Materials and Methods

Subjects

Peripheral blood samples (n=10) from healthy adult volunteers, aged 20 – 40 years, were used for study. All samples were obtained following informed consent under approval from the Vanderbilt University Medical Center Institutional Review Board.

Isolation of naïve and memory B cells from blood

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll-Hypaque density gradient centrifugation, then stained for 30 minutes at 4 °C in the dark using fluorescent conjugated mouse anti-human antibodies, including anti-CD19-PE-Cy7, anti-IgD-PE, anti-CD27-APC, anti-CD3/CD14-APC-Cy7 (Beckton Dickinson, San Jose, CA). Cells were processed immediately for flow cytometric analysis and cell sorting using a FACS Aria cytometer (Beckton Dickinson). Cells expressing CD3 or CD14 (T cell or monocyte markers) were excluded from sorting. After each experiment, a portion of the sorted sample was analyzed to determine the post-sort purity. All sorted naïve (CD19⁺IgD⁺CD27⁻) and memory (CD19⁺IgD⁺CD27⁺ or CD19⁺IgD⁻CD27⁺) B cell samples exhibited a >95% purity. Data analysis was performed using FlowJo software, version 6.1 or above (Tree Star, Inc., Ashland, OR). Representative sorting data are shown in Figure 1A.

Expansion of single B cells into clones

We used a culture system as previously described for the expansion of single B cells into clones (Weitkamp et al., 2003b). Briefly, 50,000 irradiated (50 Gy) EL-4-B5 mouse thymoma cells (kindly provided by Dr. R.H. Zubler) per well of 96-well culture plates were used as feeder cells immediately following single-cell isolation of B cells. A combination of 100 U recombinant human IL-2, 5 ng/ml PMA, and 5% (v/v) of supernatant from pokeweed mitogen-activated human T cells (T cell replacing factor) was added. The culture plates were incubated for 7 days at 37 °C in an atmosphere of 8% CO₂. After 7 days, we removed 100 µl of supernatant and added 10,000 irradiated (50 Gy) fibroblastic L cells stably transfected with human CD154 (CD40L) to each well. This cell line was kindly provided by DNAX via the ATCC (CRL 12095). We also added 5 ng/ml recombinant human IL-4 in addition to the B cell culture media described above. The cultures were kept for another 2 weeks with a second addition of CD154 fibroblasts, cytokines, PMA and T cell replacing factor on day 14. Prior to RNA isolation on day 21, human Ig capture ELISA was performed to identify the B cell clones that were secreting antibodies on day 14.

Determination of isotypes of Ab produced by single B cell-derived clones

We used a human Ig capture ELISA for determining the isotype of the Ab produced by single B cell-derived clones. Un-conjugated goat anti-human Ig (H&L) antibody (Southern Biotechnology, Birmingham, AL), diluted 1:1000 in sodium carbonate buffer was used as capture antibody. Alkaline phosphatase-labeled goat anti-human IgM, IgG or IgA antibody (Southern Biotechnology) diluted 1:1,000 in sodium carbonate buffer was used separately as secondary Ab in the replicate assays. Purified human IgM, IgG, or IgA (Bioscience

International, Saco, Maine) was used as positive control, and culture supernatant from wells lacking B cells but containing all other medium additives was used as negative control.

RNA extraction and RT-PCR amplification

We used an oligo-dT based mRNA capture kit to isolate mRNA from the suspension generated by lysis of cells in the single B-cell-derived clones secreting human Ig. We used a single-tube strategy for reverse transcription (RT) PCR amplification of the V_H region (Titan One Tube RT-PCR System, Roche Diagnostics, Mannheim, Germany). The pooled PCR primer mixture that was designed to amplify antibody genes from all antibody gene families was described previously (Weitkamp et al., 2003b).

DNA sequence analysis

We ligated gel-extracted PCR products into a TA cloning vector (Promega, Madison, WI), generated bacterial clones, and purified plasmid DNA from overnight bacterial cultures. Plasmid DNA was digested with restriction enzymes to identify clones with proper ligation. The nucleotide sequence of plasmid DNAs that contained a V_H insert was determined using vector-specific primers and an automated DNA sequencer (Applied Biosystems, Foster City, CA).

Criteria for the analysis of antibody sequences

We analyzed V_H region sequences by comparison with the international ImMunoGeneTics (IMGT) information system database (<http://imgt.cines.fr:8104>) (Ruiz et al., 2000). The characteristics of individual sequences for V_H regions are shown in the online supplemental material (Supplemental Tables I–VI). All sequences were submitted to GenBank (225 sequences, accession numbers **DQ535261** through **DQ535485**). We characterized somatic mutations as replacement (R) or silent (S) mutations by comparing variable region sequences with the corresponding IMGT germ-line sequences. The first 24 nucleotides of FR1 were encoded by PCR primers and therefore were not analyzed for mutations. Hotspot mutations were identified by comparing the nucleotide sequences surrounding mutations with known hotspot motifs (DGYW and its complement WRCH; or WA and its complement TW).

Statistical analysis

Chi-square tests, with p-values computed using Monte Carlo simulation with 500,000 replications, were used to compare proportions. Kruskal-Wallis tests were used to compare continuous variables (*e.g.*, frequency of mutations, CDR3 lengths, numbers of insertions). The unique donor exclusion method was used for examining the influence of inter-individual heterogeneity on antibody repertoire analysis of V_H, D, and J_H family and gene segments usage.

Results

Frequency of circulating naïve and memory B cells in healthy blood donors

We examined the frequency of circulating naïve and memory B cells in peripheral blood from 10 healthy blood donors. The mean frequency of B cells with naïve phenotype in these donors was 68.6% (range 27.4–89.4%). A mean of 15.1% (range 3.68–49.2%) of circulating B cells in our analysis were IgD⁺CD27⁺ memory B cells, and a mean of 13.9% (range 5.04–21.5%) of circulating B cells were IgD[−]CD27⁺ memory B cells.

Isotypes of Ab produced by single B cell clones were uniformly IgM in the naïve and IgD⁺CD27⁺ memory B cells, and were IgM, IgG or IgA in the IgD[−]CD27⁺ memory B cells

Isotypes of Ab produced by single B cell clones were determined for 80 IgD⁺CD27[−] naïve B cells, 69 IgD⁺CD27⁺ memory B cells and 80 IgD[−]CD27⁺ memory B cells clones. As shown

in Figure 1B, more than 95% of IgD⁺CD27⁻ naïve B cells and IgD⁺CD27⁺ memory B cells clones produced IgM. In contrast, the IgD⁻CD27⁺ memory B cell clones produced IgM, IgG or IgA (19%, 39%, or 36%, respectively)(Figure 1B).

The V_H antibody repertoires in circulating naïve and memory B cells were all V_H3 dominant

All statistical analyses performed here assumed independence between cells (*i.e.*, ignored any correlation that might exist between measurements taken on different cells from the same individual). It was reasonable to assume the independence of individual cells, as analysis of the V-D-J combinations revealed that none of the recombined variable regions were clonally related. We determined the nucleotide sequences of V_H gene segments from 77 naïve B cells, 63 IgD⁺CD27⁺ memory B cells and 85 IgD⁻CD27⁺ memory B cell clones isolated from seven individuals (Figure 2A) (See supplemental tables for number of Ig heavy chain sequences and corresponding gene segments analyzed from each donor). 51% of V_H gene segments in IgD⁺CD27⁻ naïve B cells, 54% of V_H gene segments in IgD⁺CD27⁺ memory B cells and 45% of V_H gene segments in IgD⁻CD27⁺ memory B cells clones belonged to the V_H3 family. The second and third most common V_H families in all B subsets cells were V_H4 and V_H1. V_H4 segments were found in 22%, 18% and 17% of IgD⁺CD27⁻ naïve B cells, IgD⁺CD27⁺ memory B cells and IgD⁻CD27⁺ memory B cells respectively. V_H1 segments were found in 21% of IgD⁺CD27⁻ naïve B cells, 18% of IgD⁺CD27⁺ memory B cells and 16% of IgD⁻CD27⁺ memory B cells. A minor trend of increased V_H5 family usage in the IgD⁺CD27⁺ and IgD⁻CD27⁺ memory B cells was noted, with approximately 3% of IgD⁺CD27⁻ naïve B cells, 8% of IgD⁺CD27⁺ memory B cells and 10% of IgD⁻CD27⁺ memory B cell clones harboring V_H5 family genes. However, we found no statistically significant difference in the distribution of V_H families among the three types of naïve and memory B cells. A unique donor exclusion method was used to evaluate the influences of inter-individual heterogeneity in V_H family gene usage analysis. Similar results were obtained in all unique donor exclusion analyses compared with the analysis result using the whole data set. Therefore, there was no apparent biased V_H family gene usage in any particular donor that influenced the overall data analysis.

The D and J_H antibody repertoires in circulating naïve and memory B cells were all D-3 and J_H-4-dominant

D and J_H family gene usages also were analyzed in the three groups of naïve and memory B cells. As shown in Figure 2B and 2C, striking similarities were found in the distributions of D and J_H family genes. D3 was identified as the most common D family in all three groups, and was found in 31% of IgD⁺CD27⁻ naïve B cells, 32% of IgD⁺CD27⁺ memory B cells and 34% of IgD⁻CD27⁺ memory B cell clones respectively. J_H4 was found to be the most commonly used J_H family in all groups. 42% of IgD⁺CD27⁻ naïve B cells, 52% of IgD⁺CD27⁺ memory B cells and 58% of IgD⁻CD27⁺ memory B cell clones used J_H4 family genes. A minor trend of increase of D6 usage was noted for IgD⁺CD27⁺ memory B cells. In addition, increased frequency of non-assignable D and J_H genes were detected in the memory B cell groups, reflecting greater frequency of somatic mutations in these cells. The unique donor exclusion method also was used to evaluate the influences of inter-individual heterogeneity in D and J_H family gene usage analysis. There was no apparent biased D or J_H family gene usage in any particular donor that influenced the overall data analysis.

Broad and similar usage of V_H, D and J_H family gene segments in circulating naïve and memory B cells

As shown in Figure 3, broad usages of V_H, D and J_H gene segments were noted in all three naïve and memory B cell groups. Of the 39 to 46 known functional V_H genes (depending on the polymorphisms present in humans), 15 different V_H3 family gene segments, 5 different V_H4 family gene segments, 5 different V_H1 family gene segments, and 1 of each the V_H5,

V_H6 , V_H2 , and V_H7 family gene segments were identified within 77 IgD^+CD27^- naïve B cell clones. Within 63 IgD^+CD27^+ memory B cell clones, 12 different V_H3 family gene segments, 5 different V_H4 family gene segments, 5 different V_H1 family gene segments, and 1 of each of the V_H5 , and V_H2 family gene segments were identified. Within 85 IgD^-CD27^+ memory B cell clones, 16 different V_H3 family gene segments, 5 different V_H4 family gene segments, 7 different V_H1 family gene segments, 2 V_H5 family gene segments, and 1 of each of the V_H6 , V_H2 , and V_H7 family gene segments were identified. Of 23 known functional D genes, 20, 18 and 21 were identified in IgD^+CD27^- naïve B cells, IgD^+CD27^+ memory B cells and IgD^-CD27^+ memory B cells respectively. All 6 known functional J_H genes were found in all groups of naïve or memory B cells. V_H , D and J_H gene segments identified in naïve or memory B cell clones were selected from across the entire gene locus and appeared to distribute in a similar and biased fashion, *i.e.*, some gene segments were overrepresented, while others were not used at all (Figure 3A, 3B and 3C). Numbers of V_H , D and J_H gene segments identified in all three naïve and memory B cell clones were similar (Figure 3D, 3E and 3F). Statistically significant differences were not found in the distributions of V_H , D or J_H family gene segments among the three types of B cells. The donor exclusion method also was used to evaluate the influences of inter-individual heterogeneity in the V_H , D or J_H family gene segments usage analysis. There was no apparent biased V_H , D or J_H family gene segments usage in any particular donor that influenced the overall data analysis.

CD19⁺IgD⁺CD27⁺ memory B cells harbor shorter CDR3s in the V_H gene segments

Analysis of heavy chain CDR3 regions suggested shorter CDR3 lengths in IgD^+CD27^+ memory B cells. As shown in Figure 4A, the CDR3 lengths for IgD^+CD27^+ memory B cells were shorter than those of IgD^+CD27^- naïve B cells ($p=0.022$). The median CDR3 length for naïve B cells was 45 bp (range from 21 to 96 bp), whereas the median CDR3 length for IgD^+CD27^+ memory B cells was 42 bp (range from 21 to 78 bp), and the median CDR3 length for IgD^-CD27^+ memory B cells was 45 bp (range from 15 to 84 bp).

Evidence for differing levels of TdT activities in the V_H -D and D- J_H junctions of naïve and memory B cells

We analyzed the V_H -D and D- J_H junctional sequences of naïve and memory B cells for evidence of the activity of insertion mechanisms. As indicated in Table I, the percentages of sequences that lacked N additions at either V_H -D or D- J_H junctions were lower in the IgD^-CD27^+ memory B cells (13.8% and 13.8%) than those in IgD^+CD27^- naïve B cells (24.3% and 21.4%) or those in IgD^+CD27^+ memory B cells (25.0% and 28.3%). Less N nucleotide additions also were noted in D- J_H junctions for IgD^+CD27^+ memory B cells. Further analyses indicated that the shorter CDR3 lengths in IgD^+CD27^+ memory B cells were a consequence of reduced numbers of N and P nucleotide insertions in both the V_H -D and D- J_H junctions. As indicated in Figure 4B, the median number of total N and P nucleotides in both the V_H -D and D- J_H junctions for IgD^+CD27^+ memory B cells was 6.5 bp (range from 0 to 34 bp), whereas the median number of total N and P nucleotides in both the V_H -D and D- J_H junctions for IgD^+CD27^- naïve B cells was 8 bp (range from 0 to 36 bp), and the median of total N and P nucleotides in both the V_H -D and D- J_H junctions for IgD^-CD27^+ memory B cells was 10 bp (range from 0 to 34 bp). The total number of N and P nucleotides in IgD^+CD27^+ memory B cells was significantly less than that in IgD^-CD27^+ memory B cells ($p = 0.04$).

Exonuclease activities in the V_H -D and D- J_H junctions of naïve and memory B cells

Examination of V_H -D and D- J_H junctions of the variable heavy chain genes revealed significant differences in the extent of exonuclease removal events between the V_H , D and J_H gene ends in B cell groups. As indicated in Table II, the number of exonuclease removals in the 3' ends of the D region was greater in IgD^+CD27^+ memory B cells than in IgD^+CD27^- naïve B cells

($p < 0.05$), while the number of exonuclease removals in the 3' ends of the D region in $\text{IgD}^-\text{CD27}^+$ memory B cells was similar to the one in $\text{IgD}^+\text{CD27}^-$ naïve B cells. In $\text{IgD}^+\text{CD27}^+$ memory B cells and $\text{IgD}^-\text{CD27}^+$ memory B cells, a similar increased level of exonuclease removals in V_H region ends was found compared with that in $\text{IgD}^+\text{CD27}^-$ naïve B cells ($p < 0.05$). In addition, exonuclease removals from V_H region ends in all three types of B cells were lower than removals from D or J_H region ends ($p < 0.0001$).

CD19⁺IgD⁺CD27⁺ memory B cells contained significantly fewer somatic mutations in V_H genes than CD19⁺IgD⁻CD27⁺ memory B cells

Over 94% of V_H genes isolated from $\text{IgD}^+\text{CD27}^-$ naïve B cells were either unmutated (72.7%) or contained a single mutation (22%). In contrast, only 6% of V_H genes isolated from $\text{IgD}^+\text{CD27}^+$ memory B cells and none of V_H genes isolated from $\text{IgD}^-\text{CD27}^+$ memory B cells were un-mutated. Consistent with a previous report about the mutation frequency of cells using a single V_H gene segment (V_H3-23), our data confirmed that the un-class-switched $\text{IgD}^+\text{CD27}^+$ memory B cells possessed significantly fewer mutations in their Ig heavy chain variable regions than $\text{IgD}^-\text{CD27}^+$ memory B cells in all segments identified in the repertoire ($p < 0.0001$). Further analysis demonstrated that mutations in both groups of memory B cells were distributed across the variable region of heavy chain genes with an increased frequency of replacement mutations, especially in the CDR1, CDR2 and FR3 regions. As expected, higher replacement/silent ratios (>3) were observed in the CDR regions of the memory B cell groups than in the FR regions (Figure 5B).

Hotspot motif analysis

Higher numbers of mutations in the context of hotspot motifs were found in the CDR regions compared with the FR regions of both sets of memory B cells. As indicated in Figure 6, the frequency of mutations within hotspot motifs and the distribution of hotspot mutations along the variable regions did not differ between $\text{IgD}^+\text{CD27}^+$ and $\text{IgD}^-\text{CD27}^+$ memory B cells. These data suggest that although the number of somatic mutations is reduced in $\text{IgD}^+\text{CD27}^+$ memory B cells compared with $\text{IgD}^-\text{CD27}^+$ memory B cells, the hotspot targeting function of the somatic mutation related machinery is fully developed in $\text{IgD}^+\text{CD27}^+$ memory B cells.

Discussion

The success of vaccination for disease prevention depends on the immunological memory that is carried by memory B and T cells. One useful approach to characterize immune memory is to study the generation, diversity and function of memory B cells that are responsible for human immunity. In this study, we analyzed human antibody variable heavy chain genes isolated simultaneously from three naïve and memory B cell groups in healthy human adult blood samples. The central findings of this study are that: memory B cells in peripheral blood share the preferential Ig gene usages with that of naïve B cells; and distinct characteristics in CDR3 regions of Ig gene rearrangements in these naïve and memory B cells indicate differential levels of activity of enzymes leading to somatic mutations and junctional diversity during the ontogeny or selection of these groups of B cells.

Antibody gene analysis at the single-cell level revealed that the V_H antibody repertoire in the three naïve and memory B cells groups were all V_H3 -dominated. We also observed a similar bias towards the D3 and J_H4 gene segment families in these three groups. When we analyzed individual V_H gene segment usages, we found further evidence for similarity of combinatorial diversity in the three groups of naïve and memory B cells. These data suggested that the antibody repertoire in human B cells is regulated tightly during the ontogeny of naïve B cells, and is not altered significantly following antigenic exposure. The broad diversity that is present in human naïve B cells and maintained in memory cells suggests a well-developed human

antibody repertoire that is established and regulated prior to, and independently of, antigen exposure.

Another important finding of this study is the delineation of fine molecular features of the Ig heavy chain genes of the recently defined un-class switched memory B cell group, IgD⁺CD27⁺ B cells. Studies from several groups have suggested unique biological functions and possibly a unique developmental pathway of this group of cells (Agematsu et al., 1998; Di Sabatino et al., 2004; Kruetzmann et al., 2003; Ma et al., 2006; Weller et al., 2004; Xiao et al., 2004; Zandvoort et al., 2001). Our data demonstrated that V_H, D and J_H gene segment usage in this type of memory B cells are broad and very similar to those of both naïve B cells and IgD⁻CD27⁺ memory B cells. Consistent with previous reports, our data indicates a significantly lower frequency of somatic mutations in the V_H genes of these cells than in IgD⁻CD27⁺ memory B cells. This finding is in line with the hypothesis that IgD⁺CD27⁺ B cells are formed in the absence of a robust T cell help environment, and may be derived from an extra-follicular pathway (Weller et al., 2001). Since the up-regulation of transcription of genes involved in somatic hypermutation, such as *activation induced cytidine deaminase* (*AID*) occurs exclusively in germinal centers in response to T cell interaction (Muramatsu et al., 1999), the lower frequency of somatic mutations in the V_H genes of IgD⁺CD27⁺ cells suggests that these B cells may mutate their antigen receptors in the absence of robust classical CD40-CD40L mediated T-B interaction characteristic of germinal center formation.

Despite the lower frequency of mutations observed in the IgD⁺CD27⁺ memory B cells, the ratio and distribution of replacement, silent or hotspot-targeted mutations across the variable regions were very similar to those in IgD⁻CD27⁺ memory B cells. Hotspot-directed mutations reflect the activity of particular error-prone DNA polymerases. As reported, somatic mutation hotspots WA/TW correspond to the error spectrum of DNA polymerase η and the consensus motifs DGYW (D=A/G/T) and its complement WRCH (H=T/C/A) are predictors of mutability in *AID*-induced immunoglobulin hypermutation (Rogozin and Diaz, 2004; Rogozin et al., 2001). Additional error-prone polymerases, such as κ , ι , μ and ζ are likely responsible for some of the observed somatic mutations that occur outside the described hotspot motifs (Faili et al., 2002; Papavasiliou and Schatz, 2002; Storb, 2001). Our findings of similar targeting features suggested that the somatic mutations that accumulated in IgD⁺CD27⁺ memory B cells were induced using similar molecular mutational machinery to that used in IgD⁻CD27⁺ memory B cells.

Our finding of shorter heavy chain CDR3 lengths in the IgD⁺CD27⁺ memory B cells provides supportive evidence of unique features in the ontogeny of these cells. Rosner *et al.* reported shorter CDR3 regions in mutated V_H6 genes rearranged to IgM constant genes when compare to un-mutated ones (Rosner et al., 2001). That study found the CDR3 region of mutated genes contained shorter V, D, J, P and N components than non-mutated genes and hypothesized that B cells bearing Ig molecules with shorter CDR3s have been selected for binding to antigen. In addition to data that is consistent with that in Rosner *et al.*'s report, our data indicated distinct characteristics of CDR3 regions in the two memory B cell groups. In our study, examination of the V_H-D and D-J_H junctions of Ig genes indicated that shorter heavy chain CDR3 lengths in the IgD⁺CD27⁺ memory B cells were due to reduced N and P nucleotide additions at both junctions. Reduced TdT-mediated extension activity and an increase of exonuclease activity at the 3' end of D genes were suggested by the nucleotide sequences of junctions in the IgD⁺CD27⁺ memory B cells compared with those of IgD⁻CD27⁺ memory B cells. The CDR3 length is determined largely in the pro-B cell stage when *recombinase activating gene* (*RAG*) 1 and 2 and *TdT* genes accomplish recombination and modification of the ends of gene segments (Fugmann et al., 2000; Komori et al., 1993; Lee et al., 2004). Therefore, these IgD⁺CD27⁺ memory B cells could be selectively derived from a more restricted naïve B cell population that harbored shorter CDR3 regions than did the circulating naïve B cells or

IgD⁻CD27⁺ memory B cells. Interestingly, studies of germinal center human tonsillar B cells, follicular lymphoma cells or synovial B cells in rheumatoid arthritis patients have demonstrated that the progress of somatic hypermutation could introduce insertions and deletions into CDR regions of immunoglobulin V genes in addition to base-pair substitutions, although at a very low frequency (Kobrin et al., 2001; Wilson et al., 1998). It is possible that the unique features we observed in the profile of junctional nucleotide insertions and deletions of V_H genes for IgD⁺CD27⁺ memory B cells were due partially to a lower frequency of somatic hypermutation events that occurred during the affinity maturation of these memory B cells.

Restriction of Ig gene usages in antigen-specific B cells have been reported in both animal and human studies of antibody responses towards a variety of antigens, ranging from hapten, carbohydrate, viral protein, Rhesus D antigens, to other self proteins (Berek and Milstein, 1987; Casadevall and Scharff, 1991; Minnerath et al., 1995; Perera et al., 2000a; Perera et al., 2000b; Zhang et al., 2006), while others reported diverse antibody responses to confined regions of foreign proteins or homologs of self proteins (Andria et al., 1990; Caton et al., 1991; Kalinke et al., 1996). We previously showed that antibody gene usages of rotavirus (RV)-specific B cells differed from that of randomly selected B cells, with a distinct bias in the usage of gene segments in the V_H1 and V_H4 families. The biased RV-specific B cell heavy chain gene repertoire was shared by both human infants and adults (Weitkamp et al., 2003a). A similar antibody gene bias was found in RV-specific IgD⁻ memory B cells expressing the intestinal homing marker $\alpha_4\beta_7$ (Weitkamp et al., 2003a; Weitkamp et al., 2005). Investigating antibody variable gene usages and molecular characteristics in naïve and memory antibody responses to a particular antigen would help to better understand the influence of affinity maturation in the maintenance, expansion, or narrowing of antigen-specific repertoires in memory B cells.

As suggested by Rosner *et al.*, shorter CDR3 regions in IgM expressing memory B cells may play a critical role in the proper interaction of antibody with antigen. Since the CDR3 loop often is situated in the center of the antigen-binding site, antibodies with shorter CDR3s may have more space in the antigen-binding pocket for the antigen to enter and make contact with the CDR1 and 2. Our observation that IgD⁻CD27⁺ isotype-switched memory B cells do not share the same molecular features of CDR3 regions with IgD⁺CD27⁺ memory B cells is interesting. The findings suggest a distinct structural determinant for Ig heavy chain CDR3 lengths that may be related to the isotypes of antibodies that are expressed in memory B cells.

In conclusion, our data revealed a highly regulated V_H gene segment repertoire that develops early in the ontogeny of human memory B cells. Our findings of distinct mutational and junctional features of antibody variable genes in IgD⁺CD27⁺ un-class switched memory B cells support the idea that these memory B cells develop or become selected in a manner distinct from that of class-switched memory B cells. Differential *TdT* mediated nucleotide addition and exonuclease activities in V_H-D and D-J_H junctions appear to contribute largely to the varied CDR3 lengths among these groups of naïve and memory B cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Agematsu K. Memory B cells and CD27. *Histol Histopathol* 2000;15:573–6. [PubMed: 10809378]
- Agematsu K, Hokibara S, Nagumo H, Komiyama A. CD27: a memory B-cell marker. *Immunol Today* 2000;21:204–6. [PubMed: 10782048]
- Agematsu K, Nagumo H, Shinozaki K, Hokibara S, Yasui K, Terada K, Kawamura N, Toba T, Nonoyama S, Ochs HD, Komiyama A. Absence of IgD-CD27(+) memory B cell population in X-linked hyper-IgM syndrome. *J Clin Invest* 1998;102:853–60. [PubMed: 9710455]
- Andria ML, Levy S, Benjamini E. Diverse VH and VL genes are used to produce antibodies against a defined protein epitope. *J Immunol* 1990;144:2614–9. [PubMed: 2108208]
- Berek C, Milstein C. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol Rev* 1987;96:23–41. [PubMed: 3298007]
- Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 2002;298:2199–202. [PubMed: 12481138]
- Camerini D, Walz G, Loenen WA, Borst J, Seed B. The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. *J Immunol* 1991;147:3165–9. [PubMed: 1655907]
- Casadevall A, Scharff MD. The mouse antibody response to infection with *Cryptococcus neoformans*: VH and VL usage in polysaccharide binding antibodies. *J Exp Med* 1991;174:151–60. [PubMed: 1676047]
- Caton AJ, Stark SE, Kavalier J, Staudt LM, Schwartz D, Gerhard W. Many variable region genes are utilized in the antibody response of BALB/c mice to the influenza virus A/PR/8/34 hemagglutinin. *J Immunol* 1991;147:1675–86. [PubMed: 1908881]
- Crotty S, Ahmed R. Immunological memory in humans. *Semin Immunol* 2004;16:197–203. [PubMed: 15130504]
- Di Sabatino A, Carsetti R, Rosado MM, Ciccocioppo R, Cazzola P, Morera R, Tinozzi FP, Tinozzi S, Corazza GR. Immunoglobulin M memory B cell decrease in inflammatory bowel disease. *Eur Rev Med Pharmacol Sci* 2004;8:199–203. [PubMed: 15638230]
- Faill A, Aoufouchi S, Flatter E, Gueranger Q, Reynaud CA, Weill JC. Induction of somatic hypermutation in immunoglobulin genes is dependent on DNA polymerase ι . *Nature* 2002;419:944–7. [PubMed: 12410315]
- Fugmann SD, Lee AI, Shockett PE, Villey IJ, Schatz DG. The RAG proteins and V(D)J recombination: complexes, ends, and transposition. *Annu Rev Immunol* 2000;18:495–527. [PubMed: 10837067]
- Kalinke U, Bucher EM, Ernst B, Oxenius A, Roost HP, Geley S, Kofler R, Zinkernagel RM, Hengartner H. The role of somatic mutation in the generation of the protective humoral immune response against vesicular stomatitis virus. *Immunity* 1996;5:639–52. [PubMed: 8986722]
- Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med* 1998;188:1679–89. [PubMed: 9802980]
- Kobrin C, Bendandi M, Kwak L. Novel secondary Ig VH gene rearrangement and in-frame Ig heavy chain complementarity-determining region III insertion/deletion variants in de novo follicular lymphoma. *J Immunol* 2001;166:2235–43. [PubMed: 11160277]
- Komori T, Okada A, Stewart V, Alt FW. Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. *Science* 1993;261:1171–5. [PubMed: 8356451]
- Kruetzmann S, Rosado MM, Weber H, Germing U, Tournilhac O, Peter HH, Berner R, Peters A, Boehm T, Plebani A, Quinti I, Carsetti R. Human immunoglobulin M memory B cells controlling *Streptococcus pneumoniae* infections are generated in the spleen. *J Exp Med* 2003;197:939–45. [PubMed: 12682112]

- Lee GS, Neiditch MB, Salus SS, Roth DB. RAG proteins shepherd double-strand breaks to a specific pathway, suppressing error-prone repair, but RAG nicking initiates homologous recombination. *Cell* 2004;117:171–84. [PubMed: 15084256]
- Liu YJ, de Bouteiller O, Arpin C, Briere F, Galibert L, Ho S, Martinez-Valdez H, Banchereau J, Lebecque S. Normal human IgD+IgM- germinal center B cells can express up to 80 mutations in the variable region of their IgD transcripts. *Immunity* 1996;4:603–13. [PubMed: 8673707]
- Ma CS, Pittaluga S, Avery DT, Hare NJ, Maric I, Klion AD, Nichols KE, Tangye SG. Selective generation of functional somatically mutated IgM+CD27+, but not Ig isotype-switched, memory B cells in X-linked lymphoproliferative disease. *J Clin Invest* 2006;116:322–33. [PubMed: 16424938]
- Minnerath JM, Mueller CM, Buron S, Jemmerson R. B lymphocyte recognition of cytochrome c: higher frequency of cells specific for self versus foreign antigen early in the immune response and V gene usage in the response to self antigen. *Eur J Immunol* 1995;25:784–91. [PubMed: 7705409]
- Muramatsu M, Sankaranand VS, Anant S, Sugai M, Kinoshita K, Davidson NO, Honjo T. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J Biol Chem* 1999;274:18470–6. [PubMed: 10373455]
- Nagumo H, Agematsu K, Shinozaki K, Hokibara S, Ito S, Takamoto M, Nikaido T, Yasui K, Uehara Y, Yachie A, Komiyama A. CD27/CD70 interaction augments IgE secretion by promoting the differentiation of memory B cells into plasma cells. *J Immunol* 1998;161:6496–502. [PubMed: 9862673]
- Papavasiliou FN, Schatz DG. Somatic hypermutation of immunoglobulin genes: merging mechanisms for genetic diversity. *Cell* 2002;109(Suppl):S35–44. [PubMed: 11983151]
- Perera WS, Moss MT, Urbaniak SJ. Comparison between hybridoma and Fab/phage anti-RhD: their V gene usage and pairings. *Dis Markers* 2000a;16:15–9. [PubMed: 11360822]
- Perera WS, Moss MT, Urbaniak SJ. V(D)J germline gene repertoire analysis of monoclonal D antibodies and the implications for D epitope specificity. *Transfusion* 2000b;40:846–55. [PubMed: 10924614]
- Prasad KV, Ao Z, Yoon Y, Wu MX, Rizk M, Jacquot S, Schlossman SF. CD27, a member of the tumor necrosis factor receptor family, induces apoptosis and binds to Siva, a proapoptotic protein. *Proc Natl Acad Sci U S A* 1997;94:6346–51. [PubMed: 9177220]
- Raman VS, Akondy RS, Rath S, Bal V, George A. Ligation of CD27 on B cells in vivo during primary immunization enhances commitment to memory B cell responses. *J Immunol* 2003;171:5876–81. [PubMed: 14634097]
- Rogozin IB, Diaz M. Cutting edge: DGYW/WRCH is a better predictor of mutability at G:C bases in Ig hypermutation than the widely accepted RGYW/WRCY motif and probably reflects a two-step activation-induced cytidine deaminase-triggered process. *J Immunol* 2004;172:3382–4. [PubMed: 15004135]
- Rogozin IB, Pavlov YI, Bebenek K, Matsuda T, Kunkel TA. Somatic mutation hotspots correlate with DNA polymerase eta error spectrum. *Nat Immunol* 2001;2:530–6. [PubMed: 11376340]
- Rosner K, Winter DB, Tarone RE, Skovgaard GL, Bohr VA, Gearhart PJ. Third complementarity-determining region of mutated VH immunoglobulin genes contains shorter V, D, J, P, and N components than non-mutated genes. *Immunology* 2001;103:179–87. [PubMed: 11412305]
- Ruiz M, Giudicelli V, Ginestoux C, Stoeck P, Robinson J, Bodmer J, Marsh SG, Bontrop R, Lemaître M, Lefranc G, Chaume D, Lefranc MP. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res* 2000;28:219–21. [PubMed: 10592230]
- Shi Y, Agematsu K, Ochs HD, Sugane K. Functional analysis of human memory B-cell subpopulations: IgD+CD27+ B cells are crucial in secondary immune response by producing high affinity IgM. *Clin Immunol* 2003;108:128–37. [PubMed: 12921759]
- Storb U. DNA polymerases in immunity: profiting from errors. *Nat Immunol* 2001;2:484–5. [PubMed: 11376332]
- Weitkamp JH, Kallewaard N, Kuschner K, Bures E, Williams JV, LaFleur B, Greenberg HB, Crowe JE Jr. Infant and adult human B cell responses to rotavirus share common immunodominant variable gene repertoires. *J Immunol* 2003a;171:4680–8. [PubMed: 14568943]
- Weitkamp JH, Kallewaard N, Kuschner K, Feigelshtock D, Feng N, Greenberg HB, Crowe JE Jr. Generation of recombinant human monoclonal antibodies to rotavirus from single antigen-specific

B cells selected with fluorescent virus-like particles. *J Immunol Methods* 2003b;275:223–37. [PubMed: 12667686]

Weitkamp JH, Kallewaard NL, Bowen AL, Lafleur BJ, Greenberg HB, Crowe JE Jr. VH1–46 is the dominant immunoglobulin heavy chain gene segment in rotavirus-specific memory B cells expressing the intestinal homing receptor alpha4beta7. *J Immunol* 2005;174:3454–60. [PubMed: 15749880]

Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, Plebani A, Kumararatne DS, Bonnet D, Tournilhac O, Tchernia G, Steiniger B, Staudt LM, Casanova JL, Reynaud CA, Weill JC. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* 2004;104:3647–54. [PubMed: 15191950]

Weller S, Faili A, Garcia C, Braun MC, Le Deist FF, de Saint Basile GG, Hermine O, Fischer A, Reynaud CA, Weill JC. CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. *Proc Natl Acad Sci U S A* 2001;98:1166–70. [PubMed: 11158612]

Wilson PC, de Bouteiller O, Liu YJ, Potter K, Banchereau J, Capra JD, Pascual V. Somatic hypermutation introduces insertions and deletions into immunoglobulin V genes. *J Exp Med* 1998;187:59–70. [PubMed: 9419211]

Xiao Y, Hendriks J, Langerak P, Jacobs H, Borst J. CD27 is acquired by primed B cells at the centroblast stage and promotes germinal center formation. *J Immunol* 2004;172:7432–41. [PubMed: 15187121]

Zandvoort A, Lodewijk ME, de Boer NK, Dammers PM, Kroese FG, Timens W. CD27 expression in the human splenic marginal zone: the infant marginal zone is populated by naive B cells. *Tissue Antigens* 2001;58:234–42. [PubMed: 11782274]

Zhang M, Zharikova D, Mozdzanowska K, Otvos L, Gerhard W. Fine specificity and sequence of antibodies directed against the ectodomain of matrix protein 2 of influenza A virus. *Mol Immunol* 2006;43:2195–206. [PubMed: 16472860]

Abbreviations

AID	activation induced cytidine deaminase
CDR	complementarity determining region
FR	framework region
Ig	immunoglobulin
PBMC	peripheral blood mononuclear cell
R	replacement mutation
RAG	recombinase activating genes
S	silent mutation
TdT	terminal deoxynucleotidyl transferase

FIGURE 1. A. Representative data from flow cytometric analyses demonstrating human naïve and memory B cells were isolated from adult blood samples with high purity

The numbers indicate the percentage of cells that were of naïve or memory phenotypes (IgD⁺CD27⁻, IgD⁺CD27⁺ or IgD⁻CD27⁺) as indicated by the oval gates. All cells plotted were gated to be CD19⁺. All post-sort samples contained >99% CD19⁺ B cells. **B. Isotypes of antibody produced by single B cell clones were determined by Ig capture ELISA.** Culture supernatants from 80 IgD⁺CD27⁻ naïve B cells, 69 IgD⁺CD27⁺ memory B cells and 80 IgD⁻CD27⁺ memory B cell clones were used for determining isotypes of antibody produced at 21 days post culture. Approximately 95% of IgD⁺CD27⁻ naïve B cells and 97% of

IgD⁺CD27⁺ memory B cell clones produced IgM. The IgD⁻CD27⁺ memory B cell clones produced IgM, IgG or IgA (19%, 39%, or 36%, respectively).

FIGURE 2. Similar V_H, D and J_H gene family utilization in circulating human naïve or memory B cells

Single circulating naïve or memory B cells from healthy blood donors were sorted, amplified in culture, and tested for antibody production by ELISA followed by sequence analysis of the expressed V_H gene segment. Frequencies of V_H, D and J_H gene family utilization in all three circulating naïve and memory B cells are presented in 2A, 2B and 2C, respectively. No difference was found in the distribution of V_H, D or J_H gene family utilization between the three types of B cells ($p > 0.5$ for all comparisons, Chi-square tests).

FIGURE 3. Similar V_H, D and J_H gene segment utilization in circulating human naïve or memory B cells

Frequencies of V_H, D and J_H gene segments utilized in three circulating naïve and memory B cells are shown in 3A, 3B and 3C. No significant difference between cell types was found for any of the V_H, D or J_H gene segment utilizations ($p > 0.2$). Numbers of V_H, D or J_H gene segments identified (used at least once within a group of cells) in all three circulating naïve and memory B cells are presented in 3D, 3E and 3F.

FIGURE 4. Shorter variable heavy chain CDR3 regions in IgD⁺CD27⁺ memory B cells

A. The CDR3 lengths for three circulating naïve and memory B cells are shown with medians and 25th and 75th percentiles (interquartile range) indicated as bars. The CDR3 lengths for IgD⁺CD27⁺ memory B cells were shorter than those of IgD⁺CD27⁻ naïve B cells, $p=0.022$. **B.** Total N and P nucleotides in both the V_H-D and D-J_H junctions of naïve and memory B cells are presented with medians and 25th and 75th percentiles indicated as bars. Total N and P nucleotides in IgD⁺CD27⁺ memory B cells were significantly less than those in IgD⁻CD27⁺ memory B cells, $p = 0.0425$.

FIGURE 5. A. Lower frequency of somatic mutations in $\text{IgD}^+\text{CD27}^+$ memory B cells

The number of mutations identified in variable heavy chain genes for circulating naïve or memory B cells is shown. Medians and 25th and 75th percentiles interquartile values are indicated. Naïve B cells possessed fewer mutations (median 0 bp) than did $\text{IgD}^+\text{CD27}^+$ (median 10 bp) or $\text{IgD}^-\text{CD27}^+$ memory B cells (median 18 bp) ($p < 0.0001$). $\text{IgD}^+\text{CD27}^+$ memory B cells possessed fewer mutations than $\text{IgD}^-\text{CD27}^+$ memory B cells ($p < 0.0001$). **B.**

Distributions of total somatic mutations and replacement mutations were similar in $\text{IgD}^+\text{CD27}^+$ and $\text{IgD}^-\text{CD27}^+$ memory B cells. Mutations in both types of memory B cells were distributed across the variable region of heavy chain genes with an increased frequency of replacement mutations (R), especially in the CDR1, CDR2 and FR3 regions. Higher

replacement mutation (R) /silent mutation (S) ratios (>3) are detected in the CDR regions of the two memory B cell groups than in the FR regions.

FIGURE 6. Frequency of mutations within hotspot motifs and the distribution of hotspot mutations along the variable regions were similar between IgD^+CD27^+ and IgD^-CD27^+ memory B cells
A. Numbers of total mutations and hotspot-targeted replacement or silent mutations within CDRs and FRs are shown. **B.** Frequency of hotspot-targeted mutations in the CDRs or FRs for the three subsets of circulating naïve and memory B cells are similar.

Table I

N nucleotide additions in the V_H-D or D-J_H junctions of the heavy chain CDR3 sequences of naïve or memory B cells.

Type of B cells	V _H -D junctions lacking additions (% of sequences)	D-J _H junctions lacking additions (% of sequences)	V _H -D N-nucleotide additions; Median (IQR)	D-J _H N-nucleotide additions; Median (IQR)
IgD ⁺ CD27 ⁻ naïve	24.3	21.4	3.0 (1.0, 6.0)	4.0 (1.0, 7.0)
IgD ⁺ CD27 ⁺ memory	25.0	28.3	3.0 (0.7, 6.0)	3.0 (0.0, 6.0)
IgD ⁻ CD27 ⁺ memory	13.8	13.8	3.0 (1.0, 7.3)	4.0 (1.0, 6.3)

* IQR=Interquartile range.

Caption: Lower percentages of sequences that lacked N additions at either V_H-D or D-J_H junctions were found in the IgD⁻CD27⁺ memory B cells than those in IgD⁺CD27⁻ naïve B cells or those in IgD⁺CD27⁺ memory B cells. Less N nucleotide additions were noted in the D-J_H junctions for IgD⁺CD27⁺ memory B cells.

Table II

Nucleotide removals in the V_H-D or D-J_H junctions of the heavy chain CDR3 sequences of naïve or memory B cells.

Type of B cells	V _H deletion; Median (IQR) [*]	5' end D deletion; Median (IQR) [*]	D segment length; Median (IQR)	3' end D deletion; Median (IQR)	J _H deletion; Median (IQR)
IgD ⁺ CD27 ⁻ naïve	0.0 (0,1.0) [†]	3.5 (1.0, 7.0)	16.0 (13.0, 20.0)	3.0 (1.0, 6.0)	4.0 (1.3, 6.0)
IgD ⁺ CD27 ⁺ memory	1.0 (0,2.0) ^{#†}	4.0 (1.8, 7.0)	14.5 (11.0, 18.0)	4.0 (2.0, 9.0) [#]	4.0 (2.8, 7.0)
IgD ⁻ CD27 ⁺ memory	1.0 (0,2.0) ^{#†}	3.0 (1.0, 7.0)	15.0 (13.0, 19.0)	4.0 (1.0, 7.0)	4.0 (1.0, 6.3)

[#] $p < 0.05$ compared to naïve cells.

[†] $p < 0.0001$ compared to removals from D or J_H region ends.

^{*} IQR=Interquartile range.

Caption: The number of exonuclease removals in the 3' ends of the D region was greater in IgD⁺CD27⁺ memory B cells. In IgD⁺CD27⁺ memory B cells and IgD⁻CD27⁺ memory B cells, a similar increased level of exonuclease removals in V_H region ends was found. Exonuclease removals from V_H region ends in all three types of B cells were lower than removals from D or J_H region ends.