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

Cuixia Tian [a], Grace K. Luskin [a], Kevin M. Dischert [a], James N. Higginbotham [a], Bryan E. Shepherd [b], James E. Crowe Jr. [a,c,*]

[a] *Department of Pediatrics, Vanderbilt University Medical Center, 21st Avenue South, Nashville, TN 37232, USA*
[b] *Department of Biostatistics, Vanderbilt University Medical Center, 21st Avenue South, Nashville, TN 37232, USA*
[c] *Department of Microbiology and Immunology, Vanderbilt University Medical Center, 21st Avenue South, Nashville, TN 37232, USA*

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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

1. 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 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

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

* Corresponding author at: Department of Pediatrics, Vanderbilt University Medical Center, T-2220 Medical Center North, 1161 21st Avenue South, Nashville, TN 37232-2905, USA. Tel.: +1 615 343 8064; fax: +1 615 343 4456.
E-mail address: james.crowe@vanderbilt.edu (J.E. Crowe Jr.).

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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. (1998) 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 three of the seven V_H gene families, V_H 1, 3, and 4. In that study, the mutation frequency of IgD^+CD27^+ memory B cells was 3.7%, 5.0%, and 5.9%, respectively, for three healthy adult donors. In a more recent report, Weller et al. (2004) 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 the V_H3–23 gene in IgD^+CD27^+ memory B cells (3.8% versus 5.7% in IgD^-CD27^+ memory B cells). 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.

2. Materials and methods

2.1. 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.

2.2. 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 min 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 FACSAria 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 Fig. 1A.

2.3. 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% CO_2. 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