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A novel human B cell subpopulation representing the initial population to express AID

Grant R. Kolar[1], Darshna Mehta[1], Rosana Pelayo[2], and J. Donald Capra[1]

[1]Program in Molecular Immunogenetics and [2]Program in Immunobiology and Cancer, Oklahoma Medical Research Foundation, Oklahoma City, OK

Corresponding author:

J. Donald Capra, M.D.
Oklahoma Medical Research Foundation
825 NE 13th St
Oklahoma City, OK 73104
(405) 271-7393
fax (405)271-8237
Donald-Capra@omrf.ouhsc.edu

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Abstract

We have identified a novel mature human B cell subpopulation in the human tonsil that has characteristics of both naïve B cells and as well as germinal center B cells including the expression of activation induced cytidine deaminase (AID) which is essential for the process of immunoglobulin somatic hypermutation and class switch recombination. These cells are clearly somatically hypermutated albeit modestly. Their phenotype ($IgD^{+}CD38^{-}CD23^{-}FSC^{hi}CD7^{+}$) is unique and suggests they may be intermediate between both naïve and germinal center cells. Morphologically they are also distinct from other B cell subpopulations. The evidence presented suggests these cells may be the founder cells of the germinal center reaction (a pro-GC cell) and may be the normal counterpart of mantle cell lymphoma.

Introduction

Peripheral B cells are widely recognized to be broadly divided into naïve, germinal center, memory, and plasma cell populations. Over a decade ago knowledge available through immunohistochemical staining of human lymphoid tissue was applied to the separation of B cells from peripheral lymphoid tissue into these and other subpopulations using flow cytometry [1,2]. Naïve B cells are recognized as $IgD^+IgM^+CD38^-CD27^-$ cells that can be further divided by expression of CD23 into Bm1 (Bm = "B mature"; $CD23^-$) and Bm2 ($CD23^+$) cells. With rare exception Ig V genes isolated from such cells are unmutated. Germinal center cells are $IgD^-CD38^+CD27^-$ and can be divided into Bm3 ($CD77^+$: centroblasts) and Bm4 ($CD77^-$: centrocytes) populations. With few exceptions cells isolated from germinal centers contain highly mutated IgV genes. While labeled numerically, it has not been conclusively established which subpopulation follows another beyond the progression of naïve B cells to become germinal center B cells before finally differentiating into an effector B cell (memory or plasma cell).

With advances in flow cytometry the number of subpopulations that can be sorted from peripheral lymphoid tissue has expanded. Pre-GC cells ($IgD^+IgM^+CD38^+$), δ–class switched cells ($IgD^+IgM^-CD38^+CD27^-$) [3], plasmablasts ($IgD^-CD38^{++}CD27^+$), memory (Bm5) ($IgD^-CD38^-CD27^+$), and marginal zone T independent ($IgD^+IgM^+CD38^-CD27^{+)}$ cells [4] among others have all been identified as distinct subpopulations of B cells in human tonsil. All of the above mentioned subpopulations of cells can now be isolated from a single tonsil using six color flow cytometry of isolated B cells. This subdivision of peripheral B cells continues to allow greater understanding of not only their function

but also of the origin, prognosis, and treatment targets for human B cell lymphomas and leukemias.

The process of affinity maturation is central to the development of high affinity antibodies. Somatic hypermutation, critical to this process, requires the presence of activation induced cytidine deaminase (AID) [5,6]. AID is also essential for class switch recombination [7-9]. While absent in naïve B cells AID expression has been repeatedly associated with mutated germinal center B cells [10,11]. The nucleic acid upon which AID acts is still a matter of debate between camps holding to the RNA hypothesis (indicating AID would itself not be the Ig somatic hypermutator but work on the transcript for another protein – reviewed in [12]) or the DNA hypothesis (AID acting directly to mutate Ig genes – the prevailing view) [13-16]. Factors surrounding its activation and regulation have yet to be fully elucidated. Identifying the initial cell population that expresses AID is therefore critical to a further understanding of the function of this molecule. Here we describe what appears to be the earliest B cell population in which AID is expressed -- an activated B cell population with many of the cell surface and molecular Ig characteristics of a naïve B cell and yet with important similarities to the cells in the germinal center.

Results

$IgD^+CD38^-CD23^-FSC^{hi}$ cells represent a novel human B cell subset

Peripheral mature naïve B cells (IgD^+CD38^-) were originally divided into two subpopulations by the expression of CD23 (Bm1 and Bm2) [1,2] (Figure 1a). In this study, in conjunction with CD23, we used forward light scatter (a measure of cell size) and the activation marker CD71 in an attempt to further subdivide this population of B cells in

the human tonsil. We identified among IgD^+CD38^- cells a population that was $CD23^-$ but FSC^{hi} ("large") instead of FSC^{lo} and was mostly $CD71^{hi}$ (85% of $IgD^+CD38^-CD23^-FSC^{hi}$ cells) (Figure 1a and 1b). For reasons expanded upon in the discussion we refer to these as pro-GC cells. This population among multiple sorts averages in size to be 0.2 to 2% of total tonsilar B lymphocytes. Previously these cells would have been classified in the Bm1 subpopulation as they are $IgD^+CD38^-CD23^-$. We saw them as distinct since Bm1 (and Bm2) cells are FSC^{lo} ("small") and $CD71^{lo}$. Germinal center cells are known to express CD71 and most have the same mean fluorescence intensity as this new subpopulation. These cells were routinely found in the tonsil but generally were not found in peripheral blood or bone marrow (data not shown).

Phenotypic analysis of a novel B cell subpopulation

In order to further characterize these cells we examined their surface phenotype (Table 1). By definition they are IgD^+ (like naïve and pre-GC cells but unlike GC cells) $CD38^-$ (like naïve and traditional memory cells but unlike pre-GC cells), $CD23^-$ (unlike Bm2) and $CD71^+$ (like pre-GC and GC cells). In addition we found that similar to Bm1 and Bm2 cells, they were $CD10^-$. As in GC cells, CD95 (Fas ligand) was expressed along with variable expression of CD77 and CD5. Unlike memory B cells $IgD^+CD38^-CD23^-FSC^{hi}CD71^+$ cells were $CD27^-$. These data distinguish this population from previously characterized human B cells.

$IgD^+CD38^-CD23^-FSC^{hi}CD71^+$ cells have Ig transcripts that are minimally mutated

Having isolated an apparently unique population of human B cells from what would previously have been considered a "naïve" pool, we sequenced 35 unique transcripts from these cells and compared them to naïve, pre-GC (IgD+IgM+CD38-),

germinal center, and memory cells all from tonsil tissue as shown in Figure 2 and Table 2. The *sine quo non* of a naïve B cell is the absence of somatic mutation, however this new cell population had significantly greater numbers of mutations per transcript (0.4 vs 1.0), yet fewer than pre-GC cells which average 3.9 mutations per sequence. This is of course, substantially lower than the level of mutation present in germinal center cells which average 14.7 mutations per transcript.. $IgD^{+}CD38^{-}CD23^{-}FSC^{hi}CD71^{+}$ cells had a replacement to silent ratio of 1.9 to 1 in line with all other mature B cell populations that are mutating with the exception of GC cells where it is substantially higher (3.9)

$IgD^{+}CD38^{-}CD23^{-}FSC^{hi}$ cells are primarily in the G1 phase of the cell cycle

Next we addressed the cell cycle stage of this new population of cells in order to understand their dynamics. Using flow cytometry, we collected cells from each major subpopulation (Bm1, Bm2, Bm3, Bm4, Bm5, and plasmablasts) in addition to $IgD^{+}CD38^{-}CD23^{-}FSC^{hi}$ cells. Consistent with their CD71 expression, an anlysis of DNA/RNA content revealed that a majority of pro-GC tonsil cells are in a G1 state at any given time. They were then fixed and stained with Pyronin Y and Hoescht 33342 dye [17-19] (Figure 3). To our knowledge this technique has not been previously reported for tonsillar B cell subpopulations. Both Bm1 and Bm2 subpopulations were primarily in the inactive G0 fraction (92% and 94%, respectively) whereas the germinal center cell subpopulation Bm4 was primarily in the early and late G1 stages (45%; for total GC subpopulations was 23%) with some cells in the S-G2-M stage (9%). The Bm3 population had the greatest percentage of cells in the S-G2-M stage (10%) with a noticeable paucity of late G1 cells indicating a relatively fast cell cycle kinetic. Memory B cells were evenly divided between G0 and G1 stages of the cell cycle. Unlike the naïve B cell subpopulations, this

new subpopulation of cells was almost entirely (83%) in the G1 stage of the cell cycle (primarily early G1).

$IgD^+CD38^-CD23^-FSC^{hi}CD71^+$ cells are larger and distinctive morphologically

The morphology of these cells in cytospins also distinguishes them among B cell subpopulations (Figure 4a) in the tonsil. They are large (>30 μm diameter) with significant amounts of homogenously stained cytoplasm, perinuclear clearing, and sizeable nuclei. Comparatively naïve B cells are small (10-20 μm diameter) with little cytoplasm and a compact nucleus. Germinal center cells are slightly larger (17-25 μm diameter) than naïve B cells and generally have more cytoplasm and a slightly larger nucleus.

Co-culture of $IgD^+CD38^-CD23^-FSC^{hi}CD71^+$ cells with T cells results in the loss of naïve and gain of germinal center markers

We used B-T cell co-culture conditions that included irradiated T cells stimulated with OKT3 along with anti-IgM for one week to stimulate $IgD^+CD38^-CD71^{lo}$ Bm1 and Bm2 cells to produce cells with the same phenotype as pro-GC cells (13.9-16.9%) compared to the same conditions without T cells (8.51-0.97%). Sorted pro-GC cells required T cells to maintain their phenotype (59.4% vs 5.56% without T cells after one week in culture) (Figure 4b). Under the same culture conditions $IgD^+CD38^-CD23^-CD71^+$ (pro-GC) cells also produced the same germinal center-like phenotype (55.9% GC-like compared to 17.2% GC-like without T cells %) (Figure 4c).

The novel B cell population expresses AID

We used a polyclonal antibody to AID in order to immunoprecipitate the protein and used a monoclonal antibody to identify AID in both nuclear and cytoplasmic

fractions by Western blot (Figure 1c). AID migrated at 27kDa. Naive cells lacked AID expression in both nucleus and cytoplasm. Germinal center cells however express AID in both compartments but primarily express AID in the nucleus. Pre-GC cells have a predominance of cytoplasmic expression. The expression of AID in $IgD^+CD38^-CD23^-CD71^+$ cells is the same between cytoplasmic and nuclear compartments. Flow cytometry using a polyclonal antibody exhibited approximately 60-70% of $IgD^+CD38^-CD23^-CD71^+$ cells were AID^+ by an MFI equivalent to germinal center but not naïve cells (Figure 1b).

In most cases only 1-2 of $IgD^+CD38^-CD23^-FSC^{hi}CD71^+$ cells are found in the germinal center

Finally, we wanted to know the distribution of this novel population of cells within tonsillar lymphoid tissue. Because they express AID we were able to use four-color immunohistochemistry to identify these cells using IgD (+), CD38 (-), and AID (+) and DAPI (Figure 5a). Using this combination of antibodies we could also distinguish naïve B cells ($IgD^+CD38^-AID^-$), germinal center B cells ($IgD^-CD38^+AID^+$), and pre-germinal center cells ($IgD^+CD38^+AID^+$). About half (48.6%) of germinal centers contained at least one $IgD^+CD38^-AID^+$ cell. We found that 72.4% of $IgD^+CD38^-AID^+$ cells were solitary and found within the germinal center (usually small ones) itself or in an interfollicular region but not in the follicular mantle.

Discussion

Over the last decade our laboratory has sequenced over 4000 Vh transcripts from the B cells of tonsil and peripheral blood and have found consistently that, although the

vast majority of naïve B cells are completely free of somatic hypermutation, there were some sequences in every pool that were mutated (Table 2). We initially considered these cells contamination but were perplexed by the consistency of these findings regardless of the hands of the experimenter. We concluded that there must be another subpopulation of B cells within the $IgD^{+}CD38^{-}$ (naïve) population.

Here we identify a novel tonsillar B cell subpopulation that has previously not been distinguished from the naïve B cell pool that explains these findings. While these cells share many cell surface markers with naïve B cells ($IgD^{+}CD38^{-}CD23^{+/-}$), they are distinguished by the presence of the activation marker CD71 and are in the early G1 phase of the cell cycle as opposed to naïve B cells which are exclusively in G0. Additionally, they are larger and unlike naïve B cells express AID and are just beginning to undergo somatic hypermutation. They do not fulfill the criteria for germinal center cells since they do not share a full complement of late activation surface markers characteristic of Bm3 and Bm4 cells (especially CD38). Consequently the identification of these cells, which seem to be intermediate between naïve and GC cells (Figure 5b), represents an important advance in our understanding of the dynamics of tonsillar B cells and the germinal center reaction.

We isolated $IgD^{+}CD38^{-}CD23^{-}CD71^{+}$ cells from several patients and, although we found a range in representation among B cells of the tonsil, it averaged close to 0.2%. While this subpopulation of cells is variable among individuals, the characteristics (activated cells with a naïve phenotype in the G1 phase of the cell cycle that are unmutated but express AID) were the same. While memory B cells are being recognized as more phenotypically diverse than previously thought [20], this novel subpopulation does

not seem to belong to a subset of cells that resembles a memory B cell. Memory B cells are typically highly mutated, often class switched, are usually $CD27^{+}$ [21], and are almost always in the G0 as well as G1 phase of the cell cycle. $IgD^{+}CD38^{-}CD23^{-}CD71^{+}$ cells do not share these characteristics. Somatic hypermutation in the naïve B cell population can now be attributed to at least two previously unrecognized populations that share the $IgD^{+}CD38^{-}$ distinction. One previously described by others is a mutated marginal zone B cells that are T cell independent [4,22], which we suspect to have a unique pattern of mutation in adults (Kolar et al. in press), and the new population of B cells described here. The removal of these two "contaminating" populations is illustrated in the changes to the somatic hypermutation profile of naïve cells in Table 2. Somatic hypermutations must be distinguished from PCR error by using calculations of background mutation rates from the constant region sequence obtained with the V region (this corrects for enzyme specific errors). Taking these corrections into account, pro-GC cells ($IgD^{+}CD38^{-}CD23^{-}CD71^{+}$ cells) had more mutations than naïve B cells and in terms of mutation frequency, place them between "true naïve" cells and pre-GC cells (see Table 2) In addition replacement to silent mutation ratios also aid in the distinction between PCR error and true somatic hypermutation since affinity maturation selects preferentially for mutations that change amino acid sequences. Hence a R/S ratio of two replacements to one silent mutation (1.9 for pro-GC) indicates mutations that have been selected as opposed to a ratio of 1 for naïve B cells. Thus, these data argue that pro-GC cells are activly mutating (albeit modestly) and are being selected.

We detected AID by Western blot and flow cytometry. We used both monoclonal and polyclonal antibodies and repeatedly detected AID in $IgD^{+}CD38^{-}CD23^{-}CD71^{+}$ cells

and germinal center whole cell lysates but not in naïve B cells. In $IgD^+CD38^-CD23^-CD71^+$ cells, AID expression was the same in cytoplasmic and nuclear compartments. A nuclear export domain is required for AID to shuttle between the nucleus and cytoplasm [23-25]. The cleavage of this domain results in the retention of AID within the nucleus and hypermutation of non-Ig targets, but its retention does not prevent somatic hypermutation of Ig genes, it only precludes class switch recombination [23,24]. Since AID expression in the nucleus and cytoplasm of $IgD^+CD38^-CD23^-CD71^+$ cells was nearly identical, the AID protein is likely to traffic between both compartments. This implies that even though the effects of the AID protein are not yet fully manifest as measured by explosive somatic hypermutation, large amounts of AID are present in the developing B cell for the first time. Taken together we conclude that $IgD^+CD38^-CD23^-CD71^+$ cells are just beginning to express AID and as such should prove useful for the study of the transition of mature naïve B cells into true germinal center cells. .

$IgD^+CD38^-CD23^-CD71^+$ cells have the phenotypic and molecular characteristics that place them between naïve and germinal center cells. Indeed $IgD^+CD38^-CD23^-CD71^+$ cells are produced in culture from naïve B cells and can be stimulated to lose IgD much like true germinal center cells. They are also very modestly somatically hypermutated with an R/S ratio reflecting the beginnings of selection for amino acid changes (these mutations are part of the process of affinity maturation). Functionally, the expression of AID places them at either a stage within the mantle zone about to enter the germinal center or within the germinal center itself. Immunohistochemistry reveals that they (and usually only one cell) are present in the germinal center. Otherwise individual cells were occasionally located in the interfollicular regions. These observations suggest

that $IgD^{+}CD38^{-}CD23^{-}CD71^{+}$ cells are a candidate to be the founder B cell of the germinal center. While clearly speculative, we propose a model in which a naïve B cell is exposed to antigen and, if its receptor is compatible and it receives appropriate T cell help in the interfollicular region, that it is activated and begins to proliferate and express AID. This cell (we favor the name "pro-GC" cell indicating its position as a cell prior to pre-GC cells and GC cells) then quickly acquires a germinal center phenotype and the process of affinity maturation begins with other interfollicular cells being pushed to the side in part forming an initial mantle zone. As cells from a maturing mantle zone are found that will recognize the key antigen in that particular germinal center, they are "picked off" and activated producing more cells with this "pro-GC" phenotype. The rarity of these cells is perhaps due to their specific phenotype that represents a snapshot of cells in a continuing process but is no less valuable as a way to specifically target the first B cell subpopulation to express AID.

The division of B cell subpopulations in peripheral lymphoid tissue has been critical for our understanding of human B cell malignancies. Prior to our understanding of the dynamics of B cell subpopulations in tissues such as the lymph node and spleen, the classification of B cell malignancies was based largely on morphology, location, and in some cases cytogenetics. Since the ability to separate normal human lymphoid tissue into distinct subpopulations and correlate morphology, phenotype, and molecular information about normal cells [1,2,26-28], our understanding of and information about the molecular biology of human B cell malignancies has grown significantly. The normal counterparts of a large number of leukemias and lymphomas have been described

(reviewed in [29]) but continue to grow as new B cell subsets are found and as new molecular data refine our understanding of the origin of these cancers.

Chronic lymphocytic leukemia cells contain Ig transcripts that can be mutated or unmutated [30-32] and has also been found to variably express AID [33]. Similarly there are reports of both unmutated and, more rarely, mutated forms of mantle cell lymphoma (MCL) [34,35]. It has not escaped our attention that this B cell malignancy shares many characteristics to the pro-GC cells described here. Phenotypically they and MCL cells are IgM+IgD+CD27-CD23- and CD10- [36]. While most MCLs are CD5+ there are reports of some that are CD5- [37] yet retain the same gene expression profile as those that have this marker. A small number of $IgD^{+}CD38^{-}CD23^{-}FSC^{hi}$ cells are CD5+ while most are CD5-. More of the pro-GC cells are CD5+ than either Bm1 or Bm2 populations and as many as 50% of GC cells were positive for CD5. MCL might simply arise from a subset of pro-GC cells or acquire CD5 immediately subsequent to transformation.

It is of interest that some MCLs express AID [35]. While it is well known that a translocation (t(11:14)) of *PRAD-1/cyclin D1* and the IgH locus results in constitutive expression of this cell cycle regulator resulting in the pathology of the disease [38,39], the stage at which this expression becomes critical is not yet clear. $IgD^{+}CD38^{-}CD23^{-}CD71^{+}$ cells may represent a stage in peripheral B cell development when the initiation of large amounts of transcription of the IgH locus first occurs in the periphery. Increased transcription would result in the disregulation of proliferation and the perpetuation of MCL. These striking similarities (cell surface phenotype, somatic hypermutation, and AID expression) between $IgD^{+}CD38^{-}CD23^{-}CD71^{+}$ cells and MCL characteristics lead us

to speculate that this novel population is a strong candidate for the normal cell counterpart of this lymphoma.

Conclusions

We describe a novel population of B cells that appears to represent cells at the onset of activation that expresses AID while not yet showing evidence of extensive somatic hypermutation (a pro-GC cell). This population may serve as a window into the beginnings of the germinal center reaction and provides a population of cells to target for the further study of expression and regulation of AID. In addition this population can be utilized targeted for the identification of immunoglobulin clones that are selected initially in response to a particular pathogen. $IgD^{+}CD38^{-}CD23^{-}CD71^{+}$ cells are therefore not only a valuable population to study in order to understand the basic process of antigen selection and the onset of somatic hypermutation, but it also may serve to be clinically important in furthering our understanding of human B cell malignancies.

Materials and Methods

Tonsil Tissue

Tonsils were obtained from either the Children's Hospital of Oklahoma in Oklahoma City, OK. The institutional review boards of the University of Oklahoma Health Sciences Center, and the Oklahoma Medical Research Foundation (OMRF) approved the use of human tissue. Protocols and informed consent procedures were provided according to Declaration of Helsinki guidelines.

Preparation of Human Cells

B lymphocytes were obtained from tissue removed during routine tonsillectomy. Total cells were collected and diluted with Hanks Balanced Salt Solution (Mediatech, Norcross, GA) and Lymphocyte Separation Media (Mediatech) was used as a density gradient to separate the mononuclear cells. After separation, the cells were washed with MACs buffer (1X PBS and 0.5% BSA) and counted. They were then enriched for B cells according to the protocol in the MACs B Cell Isolation Kit (Miltenyi Biotec, Auburn, CA).

After separation, the B lymphocytes were stained with various antibodies for flow cytometry using a MoFlo (Cytomation, Fort Collins, CO) cell sorter. Enriched B cells were sorted into the following populations: $IgD^{+}/CD38^{-}/CD23^{-}/IgM^{+}/CD27^{-}/FSC^{lo}$ (Bm1), $IgD^{+}/CD38^{-}/CD23^{+}/IgM^{+}/CD27^{-}/FSC^{lo}$ (Bm2), $IgD^{+}/CD38/IgM^{+}/CD27^{-}/CD23^{-}/FSC^{hi}$ (Bm2.2), $IgD^{-}/CD38^{+}/IgM^{-}/CD27^{-}/CD77^{+}$ (Bm3), $IgD^{-}/CD38^{+}/IgM^{-}/CD27^{-}/CD77^{-}$ (Bm4), $IgD^{-}/CD38^{-}/CD27^{+}/IgM^{+/-}$ (Bm5 – memory), or $IgD^{-}/CD38^{++}/CD27^{+}/IgM^{+/-}$ (plasmablast – PB) cells.

Antibodies for Flow Cytometry

Antibodies were either biotinylated or conjugated for sorting with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), tri-color (TC), or APC-Cyanine (APC-Cy5.5) or PE-Texas Red (ECD): anti-CD77 (clone 5B5), anti CD5 (L17F12), anti-CD10 (H110a), anti-CD39 (TU66), anti-CD40 (5C3), anti-CD95 (DX2) (Beckton Dickinson, San Jose, CA), anti-CD23 (clone 9P25) (Immunotech, France), anti-IgM (clone SA-DA4) (Southern Biotech, Birmingham, AL) anti-IgD (clone IA6-2) (San

Jose, CA) anti-CD38 (clone HIT 2), anti-CD19 (clone SJ25-C1), anti-CD27 (clone CLB-27/1), anti-CD4 (clone S3.5), anti-CD71 (clone T56/14), anti-CD20 (H147), anti-CD21 (BU32), anti-CD44 (MEM-85), anti-CD69 (CH/4), anti-CD80 (MEM-233), anti-CD86 (BU63), anti-HLA-DR (TU36) from Caltag Laboratories (Burlingame, CA). The biotinylated antibodies were stained with a secondary step using Streptavidin Red 613 (Caltag Laboratories, Burlingame, CA).

Cloning and Sequencing of Ig VH4 gene segment cDNAs

After collection of Bm2.2 or specifically AID expressing Bm2.2 cells, they were centrifuged and lysed using lysis buffer and prepared for isolation of RNA according to the protocol for the RNAqueous Micro RNA Isolation Kit (Ambion, Austin, TX). RNA was converted to cDNA by reverse transcription and amplified by PCR using gene specific primers [40] according to the protocol for the Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA). Products of RT-PCR were extracted from agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA), ligated, and cloned using the Qiagen PCR Cloning Kit (Qiagen, Valencia, CA). Plasmids from individual bacterial colonies were purified using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The purified plasmid DNAs were submitted to the OMRF DNA Sequencing Facility.

Giemsa Staining

Sorted B-cell populations: Bm1, Bm2, Bm2.2 ($CD71^+$), Bm3, Bm4, Bm5, and PB, were cyto-centrifuged for 5 min at 400 rpm onto microscope slides, air dried, and

fixed in methanol for 5-7 min at room temperature. The slides were incubated according to protocol with Giemsa stain (Sigma –Aldrich, St. Louis, MO) diluted 1:20 with distilled water for 30mins – 1hr at room temperature and then washed with distilled water. Images were captured using a Zeiss Standard light microscope with a 40x plan objective using a Canon Digital Rebel XT camera back. Brightness and contrast was globally adjusted to the same level for each image using Adobe Photoshop software.

Cell Cycle Fractionation with Hoechst and Pyronin Y

After B-cell isolation, cells were enriched with IgD by magnetic cell sorting and further stained to isolate populations for cell cycle analysis. IgD^+ cells were stained with anti-human CD38, anti-human CD27, and anti-human CD23 to obtain Bm1, Bm2, and Bm2.2 (without CD71 pre-selection). IgD^- cells were sorted into germinal center (GC), Bm3, and Bm4 populations.

A combination of Hoechst 33342 (Hst) and pyronin Y (PY) was used for the differential staining of cellular RNA and DNA as described elsewhere [17]. Briefly, the isolated B cell populations were fixed in 70% ethanol overnight, then resuspended in a solution of 2µg/ml Hst (Molecular Probes, Eugene, OR) and 4µg/ml PY (Polysciences, Warrington, PA) and measured by flow cytometry on a MoFlo equipped with UV-laser (DakoCytomation, Ft. Collins, CO). Since RNA staining with PY yields a continuous histogram without demarcation between positive and negative cells [19], an arbitrary analysis window comprising about 90% of Bm1 and Bm2 subpopulations displaying minimal PY staining was used to designate the G0 fraction in all experiments.

B-Cell – T-cell Co-Cultures

The sort purified B-cell populations Bm1, Bm2, and Bm2.2 ($CD71^+$) were cultured with autologous irradiated T cells that were $CD3^+/CD4^+$. A 96-well round bottom tissue culture plate was first coated with an anti-mouse IgG/PBS mix, diluted 1:5000, for 2 hours at 37°C. After two hours, the plate was washed and coated with a 1:5 dilution of OKT3 cell supernatant in RPMI (with 10% FCS, antibiotic-antimycotic, sodium pyruvate and L-glycine) for 2 hours to overnight at 37°C. The wells were filled with 100µL of RPMI and B-cells at 1.0×10^4/well were adjusted at a volume of 10µl/well with 1.0×10^5 at 10µl/well of irradiated T cells (irradiated at 4000 rads) in a total volume of 200µl. The control wells contained only B cells with no T cells. Diluted (1:100) anti-IgM Fab was added at 2µl/well to all wells except the control wells. The cells were incubated at 37 °C for 1 week. After 1 week the similar cell populations were pooled and analyzed using a FACs Calibur with anti-human CD27, anti-human CD23, anti-human CD38, anti-human IgD, and anti-human CD71.

Western Blot

One hundred thousand cells were isolated by flow cytometry from each population and lysed with mild detergent (1% Igepal CA-630, 0.4% sodium deoxycholate, 10mM Tris-HCl (pH = 8.0), 0.1% SDS, 5mM EDTA) for 5 minutes on ice. Lysis material was centrifuged for 5 minutes at 14,000 rpm and cytoplasmic supernatants transferred to clean tubes. Nuclear pellets were resuspended in detergent solution. AID (sc-14680 goat polyclonal antibody, Santa Cruz Biochemical, Santa Cruz, CA) was added to nuclear and cytoplasmic fractions and immunoprecipitated for 1-2 hours at 4°C with

constant tumbling. Pansorbin was added to each sample and tumbled for 1 hour at 4°C. Samples were sedimented and pellets washed with detergent solution, then suspended in 2X sample buffer, mixed, and boiled. Samples were run on a 12% acrylamide gel and transferred to PVDF membrane (Millipore Immobilon-P). Membranes were blocked for one hour in 1% BSA in TTBS buffer and incubated in primary antibody (AID mAb, mouse, #4975 (Cell Signaling Technology, Danvers, MA) 1:1000 in TTBS) overnight at 4°C with gentle shaking. Membranes were then incubated in secondary antibody (biotin anti-mouse, (Vector Labs, Burlingame, CA) 1:1000 in TTBS) for 1 hour at room temperature. Membranes were then incubated with HRP-Streptavidin (Vector Labs) 1:10,000 in TTBS) for 30 minutes at room temperature. ECL reagents (Amersham, Piscataway, NJ) were applied to the membrane and visualized using a LumiImager (Roche, Indianapolis, IN). Analysis was performed using IPLab software (Scanalytics, Rockville, MD).

Immunohistochemistry

Human tonsil tissue was fixed with 4% paraformaldehyde for 1.5 hours, rinsed, and incubated at 4° C overnight in 30% sucrose. Tissue was embedded in OCT mounting media (Bayer Corporation, Pittsburgh, PA) and 5 micrometer sections were mounted on charged glass slides. Tissue was blocked with normal donkey serum and stained with mouse anti-human IgD (LE 2) (Serotec, Oxford, UK), rabbit anti-human CD38 (Anaspec, San Jose, CA), goat anti-human AID (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies: Rhodamine-conjugated donkey anti-mouse IgG, Cy5-conjugated donkey anti-rabbit IgG (both Jackson ImmunoResearch, West Grove, PA),

and Alexa Flour 488 donkey anti-goat IgG, (Molecular Probes, Eugene, OR). DAPI: counterstain was used to visualize nuclei. For imaging a Zeiss LSM 510 confocal with META, C-apochromat 40x (N.A. 1.2) water immersion objective using PMT detectors was used. Adjustments for color balance were globally applied to images using Aperature Software (Apple Computer, Inc., Sao Palo, CA).

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1. Pascual V, Liu YJ, Magalski A, de Bouteiller O, Banchereau J, Capra JD. Analysis of somatic mutation in five B cell subsets of human tonsil. J Exp Med. 1994;180:329-339.
2. Liu YJ, de Bouteiller O, Arpin C, Durand I, Banchereau J. Five human mature B cell subsets. Adv Exp Med Biol. 1994;355:289-296.
3. Zheng NY, Wilson K, Wang X, et al. Human immunoglobulin selection associated with class-switch and possible tolerogenic origins for C[delta] class-switched B cells. J Clin Invest. 2004;113:1188-1201.
4. Weller S, Faili A, Garcia C, et al. CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans. Proc Natl Acad Sci U S A. 2001;98:1166-1170.
5. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000;102:553-563.
6. Yoshikawa K, Okazaki IM, Eto T, et al. AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. Science. 2002;296:2033-2036.
7. Honjo T, Kinoshita K, Muramatsu M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu Rev Immunol. 2002;20:165-96.:165-196.
8. Nagaoka H, Muramatsu M, Yamamura N, Kinoshita K, Honjo T. Activation-induced deaminase (AID)-directed hypermutation in the immunoglobulin Smu region: implication of AID involvement in a common step of class switch recombination and somatic hypermutation. J Exp Med. 2002;195:529-534.
9. Okazaki IM, Kinoshita K, Muramatsu M, Yoshikawa K, Honjo T. The AID enzyme induces class switch recombination in fibroblasts. Nature. 2002;416:340-345.
10. Greeve J, Philipsen A, Krause K, et al. Expression of activation-induced cytidine deaminase in human B-cell non-Hodgkin lymphomas. Blood. 2003;101:3574-3580.
11. Smit LA, Bende RJ, Aten J, Guikema JE, Aarts WM, van Noesel CJ. Expression of activation-induced cytidine deaminase is confined to B-cell non-Hodgkin's lymphomas of germinal-center phenotype. Cancer Res. 2003;63:3894-3898.
12. Honjo T. Does AID need another aid? Nat Immunol. 2002;3:800-801.
13. Faili A, Aoufouchi S, Gueranger Q, et al. AID-dependent somatic hypermutation occurs as a DNA single-strand event in the BL2 cell line. Nat Immunol. 2002;3:815-821.
14. Reynaud CA, Aoufouchi S, Faili A, Weill JC. What role for AID: mutator, or assembler of the immunoglobulin mutasome? Nat Immunol. 2003;4:631-638.
15. Wu X, Feng J, Komori A, Kim EC, Zan H, Casali P. Immunoglobulin somatic hypermutation: double-strand DNA breaks, AID and error-prone DNA repair. J Clin Immunol. 2003;23:235-246.
16. Dickerson SK, Market E, Besmer E, Papavasiliou FN. AID mediates hypermutation by deaminating single stranded DNA. J Exp Med. 2003;197:1291-1296.
17. Darzynkiewicz Z, Kapuscinski J, Traganos F, Crissman HA. Application of pyronin Y(G) in cytochemistry of nucleic acids. Cytometry. 1987;8:138-145.
18. Crissman HA, Darzynkiewicz Z, Tobey RA, Steinkamp JA. Correlated measurements of DNA, RNA, and protein in individual cells by flow cytometry. Science. 1985;228:1321-1324.

19. Gothot A, Pyatt R, McMahel J, Rice S, Srour EF. Functional heterogeneity of human CD34(+) cells isolated in subcompartments of the G0 /G1 phase of the cell cycle. Blood. 1997;90:4384-4393.
20. Ehrhardt GR, Hsu JT, Gartland L, et al. Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. J Exp Med. 2005;202:783-791.
21. 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;188:1679-1689.
22. Weller S, Braun MC, Tan BK, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood. 2004;104:3647-3654.
23. McBride KM, Barreto V, Ramiro AR, Stavropoulos P, Nussenzweig MC. Somatic hypermutation is limited by CRM1-dependent nuclear export of activation-induced deaminase. J Exp Med. 2004;199:1235-1244.
24. Brar SS, Watson M, Diaz M. Activation-induced cytosine deaminase (AID) is actively exported out of the nucleus but retained by the induction of DNA breaks. J Biol Chem. 2004;279:26395-26401.
25. Ito S, Nagaoka H, Shinkura R, et al. Activation-induced cytidine deaminase shuttles between nucleus and cytoplasm like apolipoprotein B mRNA editing catalytic polypeptide 1. Proc Natl Acad Sci U S A. 2004;101:1975-1980.
26. Frazer JK, LeGros J, de BO, et al. Identification and cloning of genes expressed by human tonsillar B lymphocyte subsets. 1997;815:316-318.
27. Liu YJ, Malisan F, de Bouteiller O, et al. Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation. Immunity. 1996;4:241-250.
28. Liu YJ, Banchereau J. Human peripheral B cell subsets. In: Weir D, Blackwell C, Herzenberg L, eds. Handbook of Experimental Immunology. Oxford, UK: Blackwell Scientific Publishing; 1996:**.
29. Pascual V, Liu YJ, Banchereau J. Normal human B cell sub-populations and their malignant counterparts. Bailleres Clin Haematol. 1997;10:525-538.
30. Gurrieri C, McGuire P, Zan H, et al. Chronic lymphocytic leukemia B cells can undergo somatic hypermutation and intraclonal immunoglobulin V(H)DJ(H) gene diversification. J Exp Med. 2002;196:629-639.
31. Naylor M, Capra JD. Mutational status of Ig V(H) genes provides clinically valuable information in B-cell chronic lymphocytic leukemia. Blood. 1999;94:1837-1839.
32. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94:1848-1854.
33. McCarthy H, Wierda WG, Barron LL, et al. High expression of activation-induced cytidine deaminase (AID) and splice variants is a distinctive feature of poor-prognosis chronic lymphocytic leukemia. Blood. 2003;101:4903-4908.
34. Thorselius M, Walsh S, Eriksson I, et al. Somatic hypermutation and V(H) gene usage in mantle cell lymphoma. Eur J Haematol. 2002;68:217-224.

35. Babbage G, Garand R, Robillard N, Zojer N, Stevenson FK, Sahota SS. Mantle cell lymphoma with t(11;14) and unmutated or mutated VH genes expresses AID and undergoes isotype switch events. Blood. 2004;103:2795-2798.
36. Dorfman DM, Pinkus GS. Distinction between small lymphocytic and mantle cell lymphoma by immunoreactivity for CD23. Mod Pathol. 1994;7:326-331.
37. Liu Z, Dong HY, Gorczyca W, et al. CD5- mantle cell lymphoma. Am J Clin Pathol. 2002;118:216-224.
38. Bosch F, Jares P, Campo E, et al. PRAD-1/cyclin D1 gene overexpression in chronic lymphoproliferative disorders: a highly specific marker of mantle cell lymphoma. Blood. 1994;84:2726-2732.
39. Espinet B, Sole F, Woessner S, et al. Translocation (11;14)(q13;q32) and preferential involvement of chromosomes 1, 2, 9, 13, and 17 in mantle cell lymphoma. Cancer Genet Cytogenet. 1999;111:92-98.
40. Kolar GR, Yokota T, Rossi MI, Nath SK, Capra JD. Human fetal, cord blood, and adult lymphocyte progenitors have similar potential for generating B cells with a diverse immunoglobulin repertoire. Blood. 2004;104:2981-2987.

Figure 1: Isolation of a novel B cell subpopulation from tonsillar B lymphocytes. IgD^+CD38^- B cells (lymphocyte gate not shown) are further separated into Bm1 (Small CD23-) and Bm2 (Small CD23+) naive B cell subpopulations along with a novel subpopulation (Large CD23-) using CD23 expression and forward scatter (FSC) (A). CD69, CD71 and AID expression is shown for each IgD^+CD38^- subpopulation as well as germinal center and memory B cells (B). Immunoprecipitation and Western blot was performed on 100,000 cells each from naïve (Bm1 and Bm2), pro-GC, pre-GC (IgD+CD38+), and GC (Bm3 and Bm4) subpopulations with polyclonal (IP) and monoclonal (blot) antibodies to AID which migrates at 27kDa (C).

Figure 2: Somatic hypermutation is present in Pro-GC cells. VH regions from immunoglobulin sequences are shown from naive, pro-GC, pre-GC, germinal center, and memory cells. VH4-34 gene segment transcripts are shown. All sequences except for those from GC and memory subpopulations were IgM+. Otherwise IgG was used.

Figure 3: Cell cycle analysis of human B cell subpopulations reveals $IgD^+CD38^-CD23^-FSC^{hi}$ ($CD71^+$) cells are in G1 phase of the cell cycle. B cell subpopulations were separated as described previously [1,2] with the addition of $IgD^+CD38^-CD23^-FSC^{hi}$ cells described in here. Cell cycle status was determined by flow cytometry of Hoechst 33342 and Pyronin Y stained populations. The diagram at the side illustrates how actively dividing cells can be resolved on the basis of RNA/DNA staining into G1 or S+G2+M fractions.

Figure 4: Morphology of $IgD^+CD38^-CD23^-CD71^+$ cells is unique among B cell subpopulation and is generated by T cell co-culture using naïve B cells. B cell subpopulations were prepared by cytospin and Giemsa staining. Cells are magnified 800x (A). Bm1, Bm2, and $IgD^+CD38^-CD23^-CD71^+$ cells were cultured with crosslinking anti-IgM and with or without OKT3 stimulated, irradiated T cells. $CD71^+CD23^-$ cells were identified after 7 days (B). Results representative of 5 experiments. $IgD^+CD38^-CD23^-CD71^+$ cells from the same co-cultures in (B) were evaluated for the loss of IgD expression (C). Percents represent the percent of cells within the given dot plot.

Figure 5: Pro-GC cells which are located in the germinal center. Anti-IgD (red), anti-AID (green), and anti-CD38 (dark blue) were used to stain frozen tonsillar sections. Lymphoid follicles were identified as naïve (red), germinal center (green), pre-germinal center (cyan appearance from combination of red, green, and dark blue), and $IgD^+CD38^-AID^+$ (yellow from combination of red and green). $IgD^+CD38^-AID^+$ cells are labeled by a white arrow. Six representative sections are shown (A). Pro-GC cells most likely exist as a transitory population between naïve and germinal center cells because of their cell surface and molecular characteristics described here. We propose that pro-GC cells lie at a critical junction in which AID expression and somatic hypermutation (SHM) begin to take place but the cell has not yet matured with a full complement of germinal center surface markers. Because of cell size and the dynamics of CD69 and CD23 expression in the tonsil subpopulations we place Bm2 prior to Bm1 in this diagram (B).

Table 1: Phenotype of 5 tonsillar B cell subpopulations*.

Marker	Population				
	Naïve	**Pro-GC**	**Pre-GC**	**GC**	**Memory**
CD5	-	-/+	-	+/-	-/+
CD10	-	-	+	+	-
CD19	+	+	+	+	+
CD20	+	+	+	++	++
CD21	+/-	+	-/+	+	+
CD23	-/+	-			
CD27	-	-	-	-	+
CD38	-	-	+	+	-
CD39	-	+/-	-	-	-/+
CD40	+	++	+	+	+
CD44	+	+	+	+	+
CD69	-/+	-	-	-	
CD71	-	+	+	+	
CD77	-	-/+	+/-	++/-	-/+
CD80	+	+++	++	++	+
CD86	+	++	+++	++	+
CD95	-	+	+	+	-
HLA-DR	+	+	+	++	+
IgD	+	+	+	-	-
IgM	-/+	-/+	-/+	-	-

*In this table relative MFIs of different levels are indicated by multiple + signs. The order of + or – signs indicates predominant population.

Table 2: Somatic Hypermutation in B cell subpopulations.

Name	**Average SHM/sequence**	**R/S ratio**
Naïve*[#]	0.7	1.8
Naïve without marginal zone and pro-GC cells**[#]	0.4	1
Pro-GC	1.0	1.9
Pre-GC[#]	3.9	1.9
Germinal Center[#]	14.7	3.9
Memory[#]	14.3	1.8

*Here naïve cells represent a population contaminated by marginal zone T independent B cells (IgM+IgD+CD27+CD38-) and pro-GC cells artificially increasing the mutation rate and R/S ratio.

**top 10% of mutated cells representing marginal zone and pro-GC cells have been removed

sequences from [3]

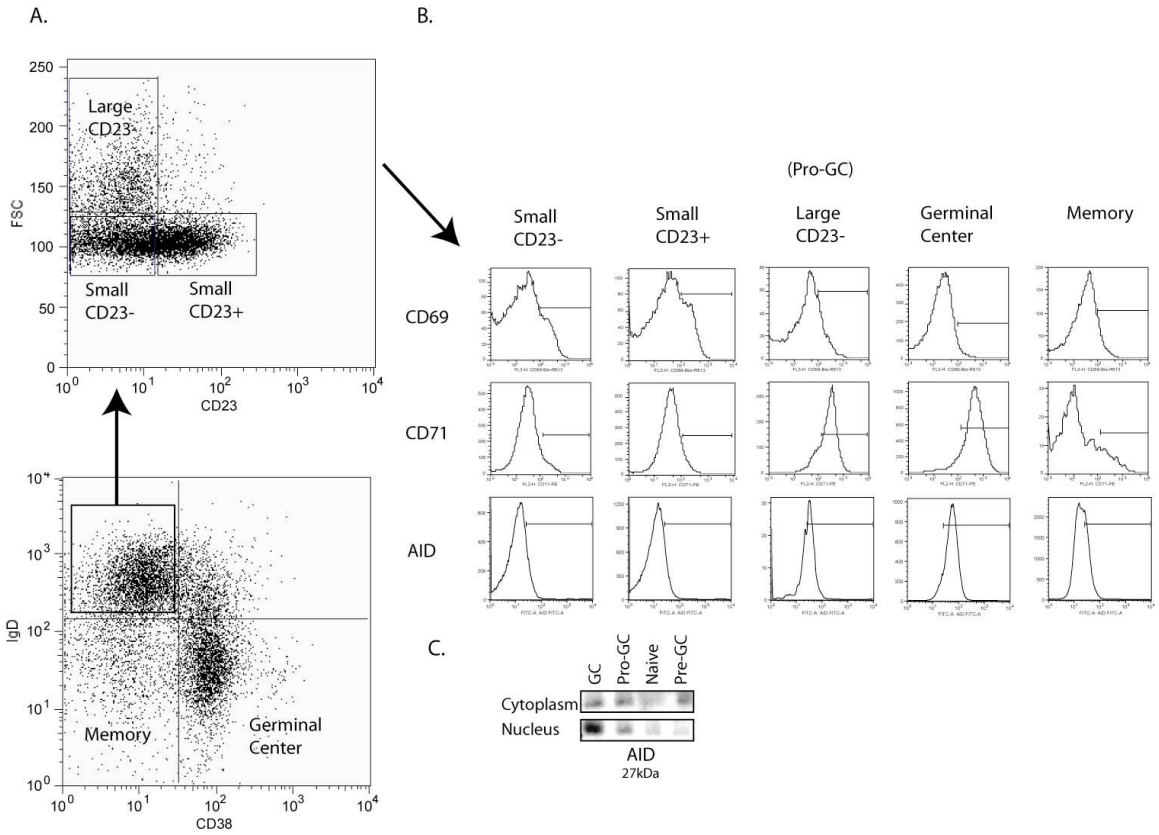


Figure 1

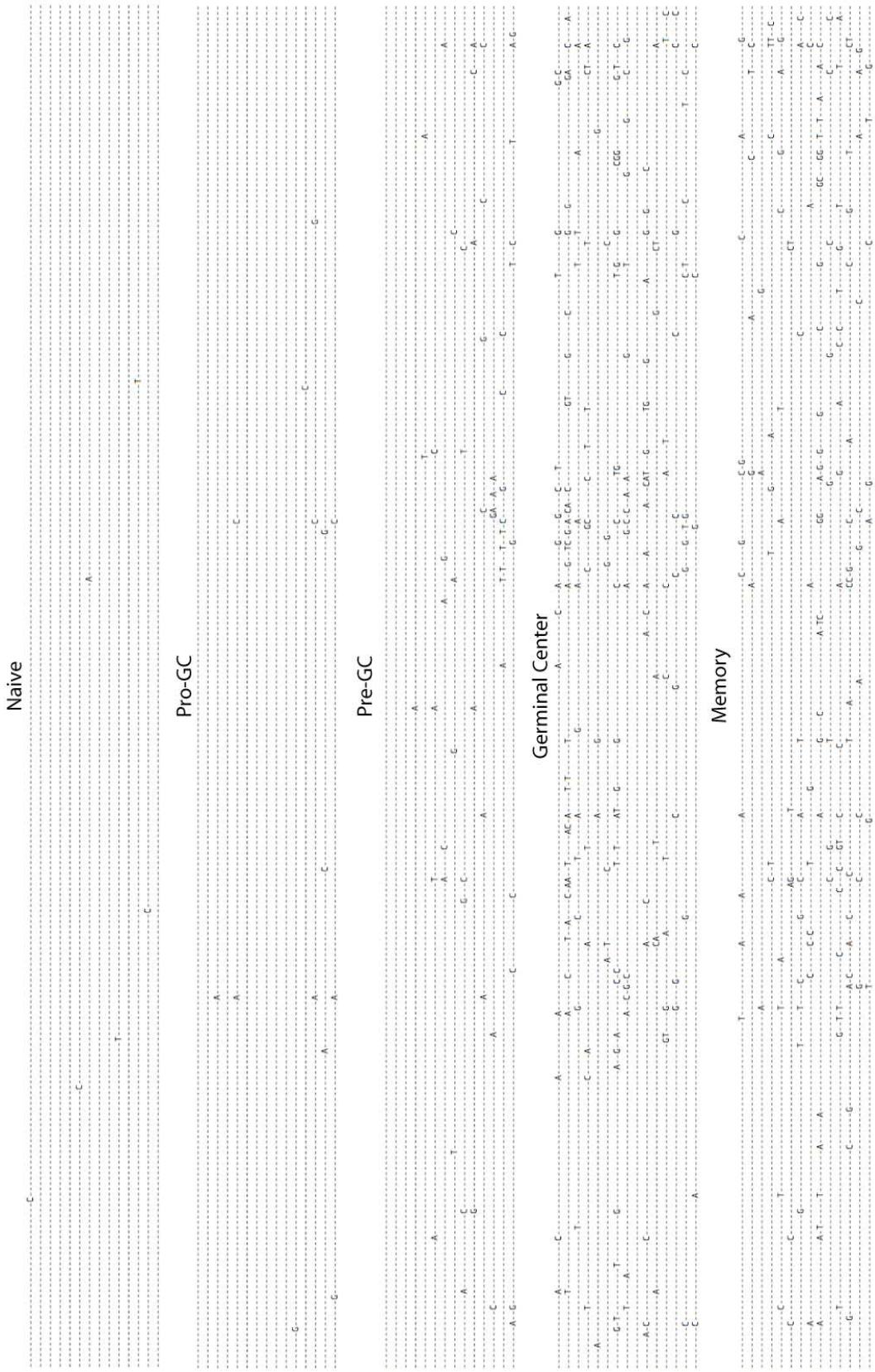


Figure 2

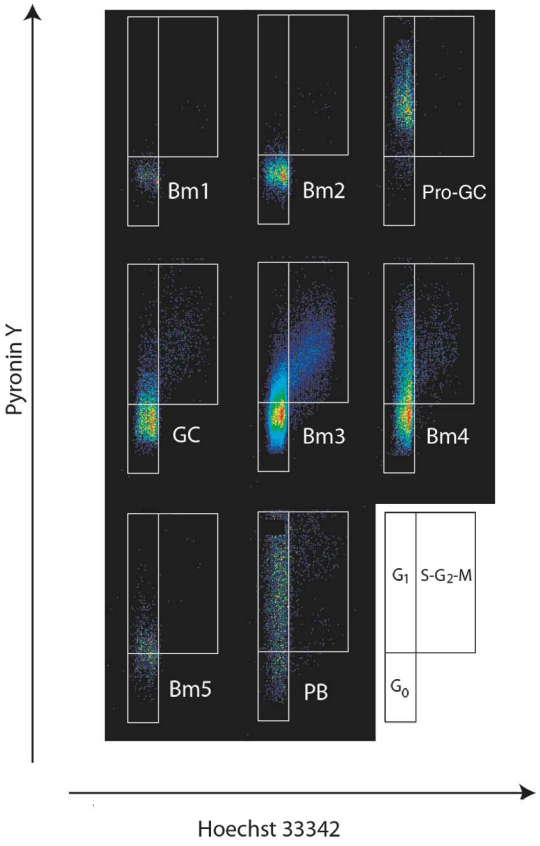
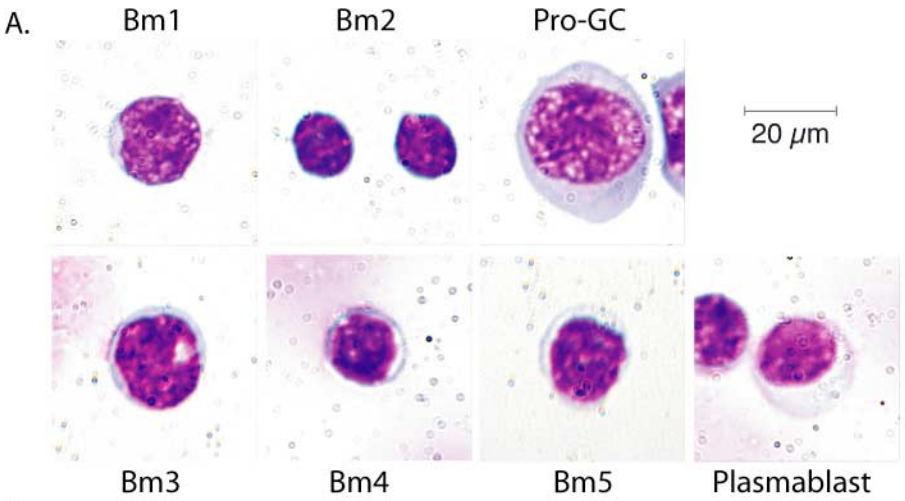
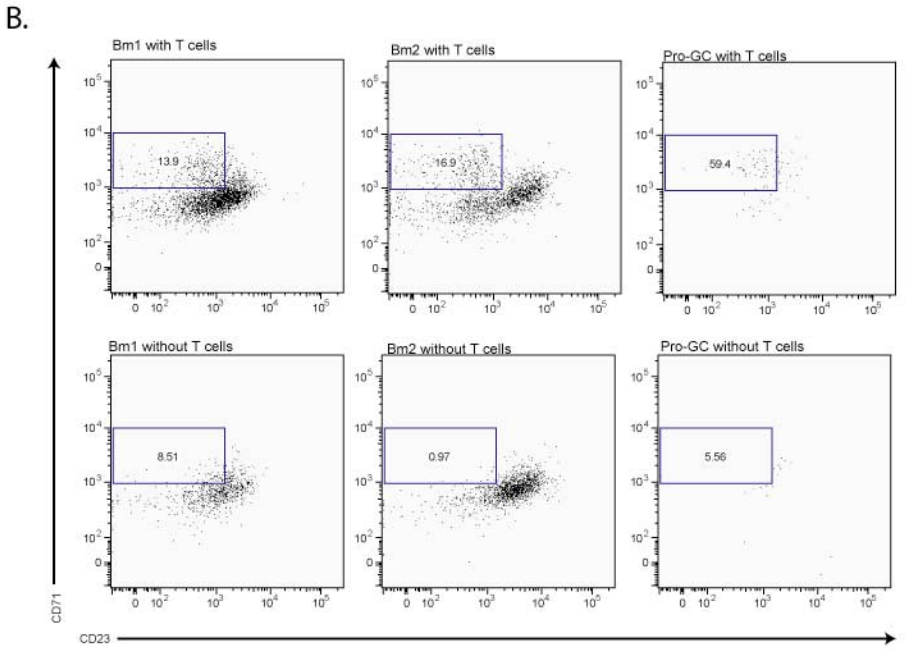


Figure 3





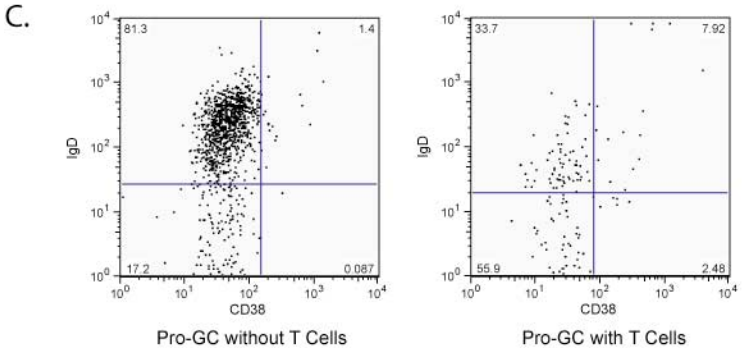


Figure 4

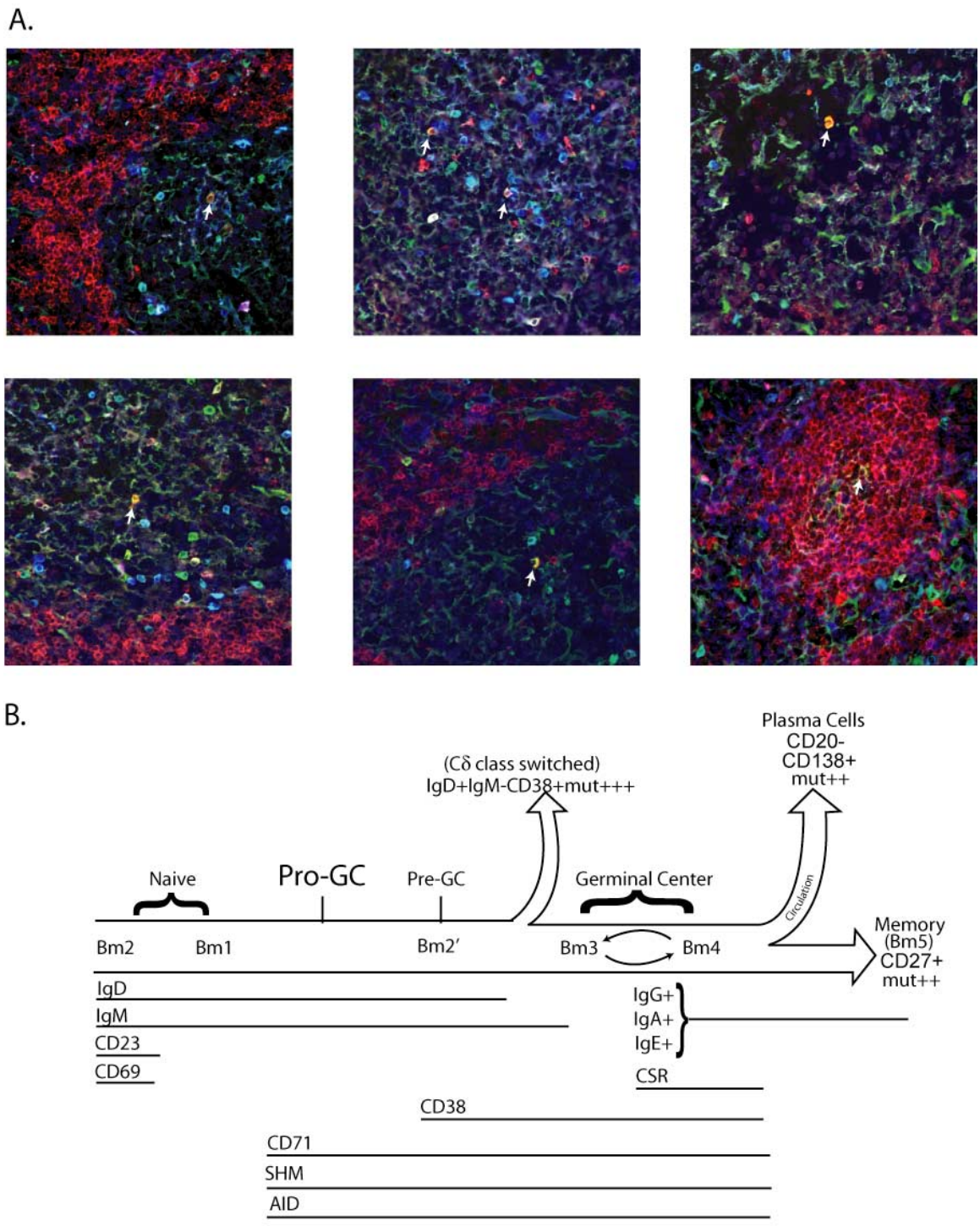


Figure 5

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A novel human B cell subpopulation representing the initial population to express AID

Grant R Kolar, Darshna Mehta, Rosana Pelayo and J. Donald Capra