CD45RO Enriches for Activated, Highly Mutated Human Germinal Center B cells

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Short Title: CD45RO Enriches for Activated, Mutated GC B cells

Key Words: B cells, germinal center, CD45RO, somatic hypermutation, CD69, immunoglobulin, human

Abbreviations used in this paper: GC, germinal center; SHM, somatic hypermutation; CSR, class switch recombination; AID, activation induced cytidine deaminase; AnV, Annexin V; IgH, immunoglobulin heavy chain; BCR, B cell receptor; FDC, follicular dendritic cell; MFI, mean fluorescence intensity
Abstract

To date, there is no consensus regarding the influence of different CD45 isoforms during peripheral B cell development. Examining correlations between surface CD45RO expression and various physiological processes ongoing during the germinal center reaction, we hypothesized that GC B cells, like T cells, that upregulate surface RO should progressively acquire phenotypes commonly associated with activated, differentiating lymphocytes. GC B cells (IgD'CD38+) were subdivided into three surface CD45RO fractions; RO-, RO+/−, and RO+. We show here that the average number of mutations per IgVH transcript increased in direct correlation with surface RO levels. Conjunctural use of RO and CD69 further delineated low/moderately and highly mutated fractions. AID mRNA was slightly reduced among RO+ GC B cells suggesting that higher mutation averages are unlikely due to elevated somatic mutation activity. Instead, RO+ GC B cells were negative for annexin V, comprised mostly (93%) of CD77+ centrocytes, and were enriched for CD69+ cells. Collectively, RO+ GC B cells occupy what seems to be a specialized niche comprised mostly of centrocytes that may be in transition between activation states. These findings are among the first to sort GC B cells into populations enriched for live mutated cells solely using a single extracellular marker.
**Introduction**

CD45, also known as the Common Leukocyte Antigen, is a protein tyrosine phosphatase that is present on the surface of all lymphoid lineage cells. Despite its nearly ubiquitous importance in proper cell activation, development, and differentiation, over the past three decades the characterization of CD45-dependent processes in lymphocytes has been confounded by at least two major observations. First, CD45 exists as several different isoforms, each with different, though not necessarily exclusive functions. Two major isoforms include the short ~180kDa CD45RO (RO) and long ~220kDa CD45RA (RA) proteins which are produced by alternative splicing of the single long RA pre-mRNA transcript. Second, the influence of a given isoform can vary between different lymphoid cell types. For example, transgenic CD45RO-only mice develop functional, mature peripheral T cells, but fail to restore their mature peripheral B cell pool. This differential effect in B and T cells prompted our investigation into the potential correlations between RO expression and key B cell biosynthetic processes executed during development and differentiation, specifically during the germinal center (GC) reaction.

The overwhelming majority of investigations regarding CD45 isoforms have focused on T cells. For example, the RA^{+}RO^{-} T cell pool is enriched for resting naïve T cells, whereas RA^{-}RO^{+} T cells are primarily activated and are comprised of memory and other effector cells. Interestingly, the intermediate RA^{+/-}RO^{+/-} fraction is enriched for primed T cells that are in bi-directional transition between the resting RO^{-} and activated RO^{+} stages. Collectively, these studies suggest that CD45 isoform expression patterns can be used to subdivide T cells into different subsets based both on their developmental stage (naïve vs. memory) and functional phenotypes such as activation, gene transcription, and protein synthesis. Unfortunately, correlations between B cell development, differentiation, and RO expression remain largely undetermined though some studies have previously
evaluated CD45 isoform expression among B cells isolated from patients with leukemia or autoimmune diseases\textsuperscript{18,19}.

Dawes et al. and Tchilian et al. have suggested that the quality and quantity of cellular activity can be significantly influenced by the level of CD45 isoform expression\textsuperscript{20,21}. RO has also been implicated in T cell deactivation and may affect signaling thresholds\textsuperscript{22,23}. Factors that potentially modify BCR-induced signaling and threshold levels are likely to affect B cell activation, selection, development, and even the propensity for disease onset. CD45 has been implicated in each of these processes\textsuperscript{18,23-26}. It is therefore important to determine for B cells, as has been done for T cells, any reproducible correlations between CD45 isoform expression and functional phenotypes. This would facilitate the identification of potential CD45-related targets for preventing or reversing anomalous cellular responses leading to the onset of B cell malignancies and autoimmunity\textsuperscript{21,27}.

Over the past two decades, our laboratory has extensively characterized events involved in human B cell development, differentiation, diversification, and selection within secondary lymphoid tissues, especially tonsils\textsuperscript{28-31}. This work has been enhanced by our ability to subdivide B cells into major groups (i.e. naïve, germinal center, and memory) according to their differential expression of key surface markers. Taking advantage of the function/activation-based discriminatory power of CD45 isoforms (demonstrated in T cells), we set out to determine whether surface RO expression, in addition to our current strategies, would enable us to further subdivide core B cell subsets into distinct fractions that are differentially engaged in one or more of the dynamic processes involved in B cell differentiation. Our investigations focused on somatic hypermutation (SHM) for several reasons. First, CD45 regulates activation induced cytidine deaminase (AID) expression\textsuperscript{32}, which is required for SHM (and class switch recombination)\textsuperscript{33-36}. In addition, SHM is unique to B cells and provides insight into the differential influence of RO between different lymphoid cell types. Finally, the number
of mutations within an immunoglobulin (Ig) sequence is easily quantified and directly correlates with
the incidence of, or length of time, a cell is exposed to the SHM machinery.

Potential correlations between RO expression and SHM were assessed by first sorting tonsil-
derived GC B cells into three pools based on their surface RO expression (RO–, RO+/–, RO+). Each
fraction was independently characterized by quantifying AID RNA transcript levels and the average
number of mutations per V_{H}4 family Ig transcript [as previously described in 37]. Results from each
RO fraction were then compared. We observed that the number of mutations increased concordantly
with RO expression. We therefore investigated whether the degree of other SHM-related processes,
including cell activation and cell division, also varied with respect to RO expression. Potential
correlations were quantified by staining RO-subdivided GC B cells with antibodies against CD69
(early activation marker, function uncertain) 38,39, CD71 (activation marker; transferrin receptor) 40,
and Ki67 (marker of proliferation; stains all non-G_{0} stages of the cell cycle) 29,41.

After documenting that surface expression of the RO and RA isoforms correlated with cellular
mRNA levels of the two major isoforms, we show here that RO expression is a powerful discriminator
of the activation state of normal human B cells. Specifically, germinal center B cells with increased
surface levels of the RO isoform were enriched for CD69 and contained the highest levels of somatic
mutations in their heavy chain transcripts. These studies document that the addition of surface RO
expression to the panel of reagents currently used to discriminate between human mature B cell
subsets provides new insights into B cell differentiation pathways and should allow further purification
of key developmental states of these critically important cells.
Materials and methods

Approval for this research was granted by the University of Oklahoma Health Science Center’s Institutional Review Board.

Lymphocyte isolation and cytometric gating

Lymphocytes were isolated from normal children’s tonsils (1-12 years) and prepared for cytometric analysis as previously described. Peripheral blood was from healthy adults. All protocols were Institutional Review Board approved. Antibodies against CD19 and CD4 were used to distinguish between B (CD19+CD4-) and T (CD19 CD4+) cells. Doublet discrimination (FSC-W vs. FSC-A and SSC-W vs. SSC-A) was applied to all analyses. B cell subsets were stained using antibodies listed in Table 1. Anti-IgD and anti-CD38 were used to identify GC (IgD-CD38+) B cells (Fig 1A). The tonsillar IgD+CD38- B cell pool is enriched for naïve B cells, which were ultimately identified by additional staining with anti-IgM and anti-CD27 (IgM+IgD-CD38-CD27-). Incubations with conjugated antibodies were for 20 minutes at 4° in the dark. Intracellular staining with Ki67 was performed by fixing cells with Fix & Perm Cell Permeabilization Kit (Invitrogen – Carlsbad, CA). Stained cells were analyzed and sorted using the LSR II (BD Biosciences - San Diego, CA), FACSaria (BD Biosciences), or MoFlo (Dako Cytomation Denmark A/S - Glostrup, Denmark) cytometers.

Lymphocyte populations were simultaneously stained with antibodies against CD45RA and CD45RO. Surface RO expression was subsequently correlated with one or more additional markers including CD69, CD71, Ki67, and Annexin V (AnV). B and T cells can express RO at similar fluorescent intensities (Fig 1B). To more accurately compare results between subjects and account for potential inter-sample variations when drawing cytometric gates, we developed a standardized gating strategy, “TB45” that uses T cell CD45 isoform expression levels to assign equivalent gates among B cells. Depicted in Figure 1B, T cell RO-, RO+-, and RO+ gating limits were applied to B cells.
from the same tonsil (PBL T cells were used for blood samples). Using this system, we reproducibly analyzed surface expression and other ex vivo phenotypes on equivalent subsets of heterogeneous lymphocyte pools isolated from multiple individuals.

RNA isolation and RT-PCR

Total RNA was isolated from sorted cells using the RNAqueous kit (Ambion – Austin, TX) according to the manufacturer’s protocol. V\textsubscript{H}4 IgM and IgG, CD45, and AID RNA transcripts were amplified using One Step RT-PCR (Qiagen - Valencia, CA). Amplification parameters were performed as previously described\textsuperscript{37,42}. Primers are listed in Table 2. Beta-actin was used to normalize RNA levels between samples for semi-quantitative RT-PCR amplifications. PCR products were gel purified using Qiaquick (Qiagen), ligated into the pDrive vector (Qiagen PCR Cloning Kit; Qiagen) and used to transform E. coli bacteria grown on agar plates containing kanamycin. Plasmid DNA was isolated and purified using the Qiagen QIAprep Spin Miniprep Kit and submitted for DNA sequencing (ABI 3730 DNA sequencer – Foster City, CA).

DNA sequence and statistical analysis

547 IgV\textsubscript{H}4 family IgM and IgG transcripts were amplified and sequenced from RO-subdivided GC B cells. V\textsubscript{H}4 family gene segment use was analyzed using National Center for Biotechnology Information IgBlast (www.ncbi.nlm.nih.gov/igblast) and IMGT/V-QUEST (http://imgt.cines.fr:8104) databases. Sequence comparisons and alignments were performed using VectorNTI. Statistical analyses including two-tailed heteroscedastic T-tests were performed using Microsoft Excel for Windows.
Results

“TB45” gating strategy standardizes CD45 isoform expression levels among lymphocytes

In this report, the accuracy of quantifying RO-associated physiological changes rests inextricably on the ability to reproducibly subdivide B cells according to their CD45 isoform expression level(s). T cells (both tonsillar and peripheral blood) have clear cytometrically defined RO− and RO+ fractions (RO+/− by non-inclusion) (Fig 1A). However, accurate assignment of RO gates for B cells is complicated by their spectral or bell-curve histogram pattern. We therefore standardized the assessment of B cell surface RO levels by assigning RO− and RO+ levels in accordance with those observed among T cells from the same tissue source. This provides a reference point enabling reliable and consistent RO quantification, even between samples collected from different subjects. Described in detail above (see Materials and methods), we term this strategy the “TB45” system which can be used for all CD45 isoforms.

Only a small fraction of tonsillar B cells express surface RO

We examined total tonsillar cells for RO and RA isoforms and found that while over 75% of T cells express modest (RO+/−) or substantial (RO+) amounts of RO, only about 15% of B cells were positive and the majority of these were only modestly positive (~10% RO+/− ; Fig 1B,C). The RO+/− and RO+ tonsillar B cell fractions appear to be constituted by a heterogeneous group of B cell subsets, of which GC B cells account for only a small percentage (19% and 12%, respectively; Fig. 1D). Therefore, high RO expression is not unique to GC B cells. Comparable percentages were observed in over 30 tonsils analyzed. RO+ B cells were predominantly observed in tonsils in contrast to peripheral blood where B cells were uniformly RO− (Fig 1B). There are both inter- and intra-subset differences in the
maximal amount of surface RO expressed among mature B cells. For example, RO⁺ cells were readily identified among GC cells, but not among tonsillar naïve B cells. In addition, the majority (93%) of all RO⁺ GC cells were CD77⁻ centrocytes (Fig 1E).

A fraction of RO⁺⁻ and RO⁺ GC B cells concomitantly downregulated surface RA in a pattern commonly associated with “transitioning” RA⁺⁻/RO⁺⁻ T cells (Fig 1B). However, it is uncertain whether increased RO expression among GC B cells correlates with differentiation (as in T cells) and receptor diversification processes, many of which initiate in response to activation-inducing stimuli and are therefore potentially influenced by CD45. We therefore investigated whether GC B cells that upregulate surface RO exhibit phenotypes consistent with diversifying (SHM) and/or activated (CD69, CD71, and Ki67) B cells.

**CD45RO mRNA levels directly correlate with surface RO in GC B cells**

CD45RO is generated by alternative splicing of the long pre-mRNA RA transcript ⁹⁻¹¹. Therefore RO and RA transcript levels are inversely related. However, to determine whether increased surface RO protein levels correlated with changes at the mRNA level (increased RO and reduced RA transcripts) in the population under study, we performed semi-quantitative RT-PCR analysis of CD45 isoform transcripts from sorted RO⁻, RO⁺⁻, and RO⁺ GC B cells. Results are shown in Figure 2A. As expected, RO mRNA and surface protein levels increased in direct proportion to one another. For example, RO mRNA was highest among surface RO⁺ cells, RO⁺⁻ cells showed a slight increase in RO mRNA, and RO⁻ cells showed only traces of RO mRNA. Again, as expected, RO⁺ cells showed a substantial decrease in RA mRNA which parallels lower RA surface expression (see Fig 1B). Finally, we found no decrease in RA mRNA in RO⁺⁻ GC B cells (relative to RO⁻ cells) even though this fraction had a lower concentration of surface RA proteins (Fig 1B).
There is a direct correlation between RO expression and somatic hypermutation among GC B cells

The hallmark phenotype of GC B cells is their prodigious rate of cell division, during which cells diversify their antigen receptors (BCR) through AID-dependent somatic hypermutation (SHM) and class switch recombination (CSR)\textsuperscript{33-36}. To determine whether SHM was more likely to occur during a given RO stage, we first compared AID transcript levels between the various RO fractions. As shown in figure 2B, AID transcripts were positively detected in each RO fraction.

The number of somatic mutations progressively accumulates over time as cells continue through cyclic stages of proliferation (incorporation of mutations) and non-proliferation (selection and eventual differentiation). Using the average number of mutations per sequence as a temporal gauge, we investigated whether surface RO expression could be used to enrich for GC B cells that are in early (germline or few mutations) or later (higher mutation averages) stages of the GC reaction. A total of 547 IgM and IgG transcripts were sequenced in order to get large enough sample sizes for a comprehensive analysis.

Results from two independent tonsils (T76 and T78) are shown in Figure 3A. For both tonsils, the average number of mutations per $V_{H}4$ sequence increased in direct proportion to surface RO expression. The trend of increased mutations among RO$^{+}$ GC B cells was repeatedly observed in both IgM and IgG sequence pools. For example, T76 averages increased from 4.2 to 11.4 (IgM) and 9.5 to 12.9 (IgG) for RO$^{-}$ vs. RO$^{+}$ cells, respectively. Statistical significance was high in each case with p-values less than 0.002 (except T78 IgM; p=0.04). Mutation averages among RO$^{+/c}$ GC B cells were significantly different from RO$^{-}$ or RO$^{+}$ values in 5 out of 8 comparisons (i.e. RO$^{+/c}$ vs. RO$^{-}$ and RO$^{+/c}$ vs. RO$^{+}$ for IgM and IgG sequences from both tonsils). Interestingly, RO$^{+/c}$ mutation averages
were closest to the averages for their respective total pools [i.e. T76 total IgM (7.9), RO+/-IgM (7.3); total IgG (11.2), RO+/- IgG (12.4)].

We next compared the percentage of unmutated sequences in each RO fraction to determine whether higher RO+ mutation averages were due to a reduced number of unmutated cells in this fraction (i.e. a greater ratio of mutated vs. unmutated sequences). Graphed in Figure 3B, the percentage of unmutated IgM sequences in RO- and RO+ fractions respectively decreased from 29% to 9.3% (T76) and 25% to 16.7% (T78). Unmutated IgG sequences were predominantly identified among RO- fractions for both tonsils. For example, all T76 RO- and RO+/- IgG+ sequences analyzed were mutated. Though 3 (n=57) unmutated RO+ IgG sequences were identified in T78, collectively (T76 + T78) unmutated IgG sequences were 3 times less likely to be RO+ (3/115) than RO- (8/102).

The near exclusion of cells containing unmutated Ig heavy chain transcripts, and small size of the RO+ pool (<10% of all GC cells) suggested that only a select group of cells upregulate surface RO at any given time. Therefore, we generated cumulative distribution graphs to determine whether higher RO+ mutation averages were due to skewing by a few sequences with extremely high mutation frequencies (Fig 3C, D). Percentages represent the number of samples that have mutations within the indicated range divided by the sample size n-value (range increases in 3 base pair increments). Arrows highlight the percentage difference between RO- and RO+ fractions at the RO+ average. RO+ GC B cells (independent of isotype) were enriched for more sequences with higher mutation averages. For example, only 5% of T76 RO-IgM+ sequences have more than 10 mutations compared to 35% of RO+IgM+ sequences, representing a 7-fold increase (Fig 3C). Note that RO+/- values are typically medial to RO- and RO+ values suggesting that mutations progressively accumulate with respect to RO level. Similar results were observed for T76 IgG sequences, with the largest
differences occurring in the lower mutation range (i.e. 95% of RO⁺ IgG sequences have 8 or more mutations, compared to only 50% of RO⁻ sequences).

**RO⁻ GC B cells express the highest level of Annexin V**

It has been reported that cells with increased autoreactive potential can have extremely high mutation averages. Lui and Arpin suggest that incorporating more mutations would increase the chances of ablating BCR auto-specificity, which if uncorrected, could result in cell death. To determine whether the RO⁺ fraction was enriched for apoptotic cells, total tonsillar B cells were stained for GC and naïve B cell subsets as before with the addition of anti-annexin V-APC (a marker of cell death). Illustrated in Figure 4A, 94.5% of all AnV⁺ GC B cells (0.6%) are RO⁻. Only 0.5% of RO⁺ GC cells were AnV⁺. The close proximity of most AnV⁺ cells to the RO⁻:RO⁺⁻ boundary suggests that the RO⁻ fraction can be further subdivided to highlight an RO⁻lo fraction. These data suggest that essentially all RO⁺ GC cells are alive and non-apoptotic. To determine whether AnV was more likely a marker of cell death or perhaps a marker expressed by differentiating cells (reported in ), CD69 mean fluorescence intensities were calculated for AnV⁻ and AnV⁺ cells within each RO fraction (Fig 4B). Due to the dearth of RO⁺AnV⁺ cells, comparisons were made by gating on the highest and lowest 5% of cells under the AnV bell-curve. Independent of RO fraction, CD69 MFI values were 3-4 fold lower in all AnV⁺ GC cells, which is more consistent with cells that are in the process of downregulating many homeostatic processes.

**An increased percentage of RO⁺ GC B cells are CD69⁺ with minor changes in CD71 expression**

GC B cells become activated as they begin to somatically mutate. We investigated whether the RO-related increase in mutations correlated with signs of increased activation, including proliferation.
GC B cells were co-stained with antibodies against RO and a panel of activation markers including CD69, CD71, and Ki67. Isotype controls were used to demark positive and negative gates for each cytometric bell curve distribution. Results are depicted in Figure 5A. Each RO fraction contained both CD69+ and CD69− GC B cells. However the highest ratio of CD69+ to CD69− cells was observed within the RO+ fraction (r= 1.0), compared to RO− (r < 0.1) and RO+/− (r < 0.1) fractions. Consistent with these findings, RO+ cells showed a 4-fold increase in CD69 MFI values compared to RO− cells (Fig 5B).

CD71 is upregulated among cells that require additional iron uptake for metabolic (and other) processes including cell differentiation and division. Similar to CD69, CD71+ and CD71− GC B cells were observed in each RO fraction. However the ratio of CD71+ cells did not significantly increase in the RO+ fraction (r = 0.5) compared to RO− (r = 0.4). RO+/− cells showed a two-fold increase (r =1.1), but comparison of CD71 MFI values revealed only a minor increase in this fraction (Fig 5B). Based on these data, RO+ cells showed increased signs of certain (CD69), but not all (i.e. CD71) types of activation compared to their RO− counterpart. We therefore assessed whether activation markers that are more directly linked with the SHM process in GC B cells were differentially expressed in relation to surface RO.

The majority of proliferating GC B cells is RO−

Cell division is a prerequisite for SHM. Ki67 is routinely used to identify proliferating lymphocytes, positively stains cells in all stages except G0, and generally parallels CD71 expression. We compared Ki67 expression between RO-sorted GC B cell fractions to determine whether proliferation (or non-proliferation) varied with respect to RO. Shown in Figure 5A, 71% of GC B cells were Ki67+ and most likely in a non-G0 stage of cell cycling (especially the 53% CD71+Ki67− double positives).
Like CD69 and CD71, Ki67+ GC B cells were observed in all three RO fractions. Both the ratio of Ki67+ to Ki67- cells (Fig 5A) and Ki67 MFI values (Fig 5B) varied little between RO fractions. These findings, combined with CD71 data render it impossible to rule out the potential existence of proliferating cells within each RO fraction. Nonetheless, nearly 70% of RO- GC B cells are Ki67+ suggesting that the majority (but not necessarily the totality) of proliferation occurs during this stage.

**Conjunct staining against CD69 and RO further subdivide GC B cells into isotype-influenced, differentially mutated subsets**

Of the activation markers surveyed, CD69 provided the greatest MFI difference between RO+ and RO- GC B cell fractions. We therefore investigated whether the combined use of anti-CD69 and anti-RO could be used to subdivide GC B cells within the same RO fraction into two pools with relatively low and relatively high mutation frequencies. GC B cells were first gated according to their surface RO expression and subsequently sorted into CD69- and CD69+ fractions (i.e. RO+CD69- and RO+CD69+, RO-CD69-, etc). Results are shown in Figure 6. Like RO, CD69 alone separated GC B cells into subsets with significantly different mutation averages (Fig 6A). Interestingly, the correlation between CD69 expression and increased mutations were inversely related for IgM and IgG V_{H}4 transcripts sequenced in this experiment. For example, sequences from CD69- IgM cells had higher mutation averages than CD69+ IgM cells (11.7 vs. 5.9, p≤0.001). In contrast, CD69- IgG cells were lower than their CD69+ counterpart (10.1 vs. 12.0, p=0.05). The same analysis (CD69- vs. CD69+) was independently applied to RO- and RO+ fractions (i.e. RO-CD69- vs. RO-CD69+). Combined use of CD69 and RO resulted in the largest difference in mutation averages ([RO+CD69- - RO-CD69-]= [16.7 - 4] = 12.7) compared to either RO+ vs. RO- (7.2) or CD69+ vs. CD69- (5.8) alone. We next performed semi-quantitative RT-PCR analyses on CD69- and CD69+ GC B cells from each RO...
fraction to determine whether surface CD69 could be used as a marker to enrich for GC B cells with higher or lower levels of AID expression (mRNA). Depicted in Figure 6B, AID was comparably expressed in CD69+ and CD69− GC B cells isolated from the same RO fraction. AID expression was inversely proportional to surface RO expression, with the highest level detected among RO− cells. These experiments show that while RO and CD69 are effective independently, when used in combination they provide the best discriminator for human GC cells that have the lowest or the highest mutation averages.

Discussion

For over a decade, we have reproducibly separated human B cells into distinct subsets including naïve, GC, and memory, each of which displayed characteristic mutation averages. However, differences were more attributable to their developmental stage such that antigen-inexperienced naïve B cells were unmutated and high affinity (SHM-dependent) memory B cells were among the most mutated. In contrast, GC B cells spanned the entire spectrum of mutations. Previous attempts using anti-CD77 (centrocyte vs. centroblast) did not reliably separate GC B cells into enriched pools of cells with low and high IgH mutation averages. Typically, centrocytes and centroblasts had virtually identical mutation averages. Here we show that the level of somatic mutation among GC B cells increased concordantly with RO upregulation. These results document for the first time that GC B cells can be reproducibly separated into pools enriched for low or higher mutation averages, based solely on an extracellular surface protein.

A significant increase in mutations was consistently observed among RO+ fractions (relative to RO−) for both IgM and IgG sequences. Cumulative distribution analyses show that average increases within each RO fraction were gradual and were not due to the over-expansion of a highly mutated
clonal family (mutation increases were observed for multiple \( V_{H4} \) family members; sequence data not shown). \( RO^+ \) fractions had both a greater percentage of cells with more mutations (as high as a 7-fold increase in some cases, Fig 3C) and a decreased number of unmutated germline sequences. The latter was especially true for the IgG pool derived from \( RO^+ \) GC cells, where in one tonsil, all 58 \( Ig_{VH} \) IgG sequences were mutated.

Like RO, CD69 also subdivided GC B cells into subpopulations with different levels of somatic mutation (though with a narrower range of difference; \( RO^{\text{only } IgM}=7.2, \ CD69^{\text{only } IgM}=5.8 \)). However, the combination of both RO and CD69 provided the greatest separation between cells bearing \( Ig_{VH4} \) transcripts with low and high mutation averages (\( RO \) and \( CD69 \) IgM = 12.7; Fig 6A). Interestingly, CD69 had no discriminatory effect on \( RO^- \) GC B cells. It is uncertain what factor(s) contributed to the inverse mutation pattern observed between CD69-subdivided \( RO^+IgM^+ \) and \( RO^+IgG^+ \) sequences (see Figure 6A). Results may point towards different influences of CD45 on AID-dependent SHM and class switch recombination. Comparable mutation averages with \( RO^+IgG^+ \) sequences render it plausible that CD69 \( RO^-IgM^+ \) GC B cells represent those that will imminently class switch and/or differentiate into post-GC B cell subsets. Alternatively, this fraction may represent highly mutated cells whose BCR confers self-specificity and are therefore targeted for deactivation during their potential transition towards anergy or deletion. Further investigation is needed to address these two points.

In preliminary efforts to uncover what force(s) are likely to contribute to differences in mutation averages between \( RO^+ \) and \( RO^- \) fractions, we investigated whether other SHM-related processes were differentially expressed with respect to RO. Three major factors critical for the initiation and persistence of SHM include AID expression (also responsible for class switch recombination), cell activation (i.e. CD69, CD71), and proliferation (access to exposed nucleic acid; Ki67)\(^ {33-36} \). AID, Ki67,
and CD71 were detected in each fraction making it impossible to rule out proliferation or SHM during any RO stage. Nonetheless, AID expression decreased slightly among RO-expressing GC B cells suggesting that at least a fraction of these cells may have terminated, or are in the process of downregulating AID-dependent processes, including SHM. The relative difference in AID expression between RO− and RO+ cells varied between tonsils (Figures 6B and 2B). Further experiments are needed to better determine whether certain cell cycle stages, particularly S-phase, predominate in a given RO fraction. Nonetheless, the majority of G0 (Ki67−) cells were RO− (Fig 5A). Preliminary analyses using forward and side scatter showed that RO+/− GC B cells were larger (FSChi) with greater internal complexity (SSC hi) than RO− cells (data not shown) which may be indicative of different stages of the cell cycle occurring during a given cell’s occupancy of RO− (resting, early stage) and RO+/− (mid-late and/or S phase) fractions. Further experiments will be required to fully understand this distinction. Previous reports concluded that mutations are incorporated during the CD77+ centroblast stage. Here we show that nearly all centroblasts (99%) were RO− or RO+/− suggesting that the majority of mutations are incorporated during one or both of these stages.

It is possible that proliferating RO+ AID+ cells may be involved in other AID-associated processes, i.e. CSR. Investigations by Liu et al., suggest that SHM precedes CSR. While this order of events may occur in most cases, it does not appear to be obligatory. First, unmutated IgG sequences, though rare, are routinely isolated. Secondly, we show that IgG transcript mutation averages continue to increase with respect to RO expression. In addition, we identified several examples of clonally related IgG sequences that had different numbers of mutations per transcript (data not shown; manuscript in preparation). Collectively, these data provide sequence-related support that GC B cells that have undergone class switch recombination can in some cases re-initiate
the somatic hypermutation process, and that CSR is unlikely to occur solely at the end of the GC reaction.

The ability to subdivide B cells into physiologically relevant levels of RO expression is central to our understanding of these data. The accuracy of B cell RO gating was evidenced by changes in CD45 isoform transcript levels. Despite a seemingly small increase in detectable surface RO levels, RO-expressing GC B cells showed increased RO transcripts, reduced RA transcripts (possibly due to increased RA mRNA splicing and/or reduced overall synthesis of the long isoform), and adopted unique physiologically relevant phenotypes. The low percentage of RO-expressing tonsillar B cells (10-20%) suggests that expression, or at least sustained expression is not required for cell survival or retention within the tonsil environment. Though readily observed in tonsils (and presumably in other lymphoid tissues), RO+ B cells were rare in peripheral blood suggesting that factors that promote RO-upregulation are either unique to, or differentially administered in tonsils.

RA+RO- resting naïve T cells upregulate surface RO (RA +/- RO +/-) in response to activation and differentiation-inducing stimuli during “transition” towards memory and effector cells. We therefore hypothesize that GC B cells that upregulate surface RO may be in the midst of transition, whether from one physiological state to the next (i.e. proliferating to non-proliferating), or differentiation to other B cell subsets. Further investigation is needed to address this issue more directly. Nonetheless, data presented in this report show that RO-expressing GC B cells, especially RO+, exhibited characteristics that are consistent with cells in transition including increased activation (CD69), enrichment for CD77+ centrocytes (poised to undergo selection), and a decreased propensity for cell death (AnV-).

In summation, by adding CD45RO (and CD69) to our current strategy for identifying human GC B cells, we introduce a “temporal-gauge” whereby cells can be separated into pools with low
(recent descendents of the founder GC B cells) and high mutation averages (distally related to founder cells evidenced by multiple cell divisions; i.e. cells mutate at a rate of 1 mutation per 3 cell divisions). It is important to note however, that surface RO was also expressed by human B cell subsets other than GC B cells. Since mutations are predominantly introduced during the GC stage, it is unlikely that RO correlates with SHM in these cells. Applying similar RO-based strategies to subdivide non-GC B cells may elucidate whether surface RO correlates with other physiologically relevant phenotypes as it does with mutation load in GC B cells.

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Author Contribution

Authors listed for this manuscript have contributed in the following manner. SM. Jacks on wrote the manuscript, designed the research project, performed the research and analyzed the data. Research was also performed by N. Harp, D. Patel, J. Zhang, S. Wilson, YJ. Kim, and C. Clanton. As the principal investigator, JD. Capra reviewed the data and final manuscript.
References


**Table 1: List of Antibodies (Cytometry)**

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<th>Antibody</th>
<th>Fluorophore</th>
<th>Company</th>
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<td>IgG</td>
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Calltag – Burlington, CA; Southern Biotech – Birmingham, AL; BD Pharmingen – San Diego, CA
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<th>GENE</th>
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<td>(V_{H}4) IgM</td>
<td>5'-ATGAAACACCTGTTGGTTCTT-3'</td>
<td>5'-CACGTTTTTTCTTTTGTGTC-3'</td>
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<tr>
<td>(V_{H}4) IgG</td>
<td>5'-ATGAAACACCTGTTGGTTCTT-3'</td>
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<td>5'-ATCCACAAGGAGTACTTGGGCTCA-3'</td>
<td>5'-GTACCACTGGCATCGTGATGGACT-3'</td>
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<tr>
<td>CD45(^a)</td>
<td>5'-GGCAAGCCCAACACTCTT-3'</td>
<td>5'-TGTTGGTGAAATGACAGCG-3'</td>
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<td>AID(^b)</td>
<td>5'-ACTTGCGGGAGGCAAAGAAGACACTCT-3'</td>
<td>5'-CAAAAGGATGCGCCGGAAGCTGCTGGAG-3'</td>
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</table>

\(^a\): Rat et al., \(^b\): Zhou
Figure 1

A

Total Tonsil B Cells
Tonsil Naive Enriched

B

Tonsil T Cells
Tonsil B Cells
PBL B Cells
Tonsil GC B Cells
Tonsil Naive B Cells

C

Tonsil Total B
Tonsil Naive B
Tonsil GC B

D

% Cells per Fraction

% GC Cells among Total
B Cells within Each Fraction

E

Total Tonsil B cells
Tonsil Naive B cells
Tonsil GC B cells
Figure 2

A

MARKER  RO⁻  RO⁺⁻  RO⁺

750
500

→ CD45RA

B

500

→ AID

500

→ BETA ACTIN
FIGURE 4
Figure 5

A

B

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Figure 6

A

![Graph showing mutation average for CD69- and CD69+ cells]

- T76: p ≤ .001
- p = .05
- p ≥ .001

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</table>

B

- MARKER
- RO-
- AID
- BETA ACTIN
CD45RO enriches for activated, highly mutated human germinal center B cells

Stephen M. Jackson, Natessa Harp, Darshna Patel, Jeffrey Zhang, Savannah Willson, Yoon J. Kim, Christian Clanton and J Donald Capra