Cutting Edge: Redox Signaling Hypersensitivity Distinguishes Human Germinal Center B Cells

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The interplay between kinase activity and phosphatase regulation is thought to determine the fate of mature B cells undergoing the germinal center (GC) reaction. In addition to BCR signaling, secondary messengers control the signaling context and help determine functional outcomes in B cells. $H_2O_2$ is the primary reactive oxygen species (ROS) produced by B cells. $H_2O_2$ amplifies BCR signaling by transiently inhibiting BCR-associated protein tyrosine phosphatases (1). $H_2O_2$ is also produced as part of innate immune responses to wounds and infection (2). However, it is not known what impact $H_2O_2$ has on healthy human B cell signaling responses and whether B cells undergoing GC reactions respond differently to $H_2O_2$.

Seconds after BCR crosslinking, a network of signaling molecules becomes activated through posttranslational modifications. As signaling directs B cells down differentiation pathways, B cells adopt well-characterized signatures defined primarily by protein expression (3), naive B cells in humans are defined by expression of CD19, CD20, and IgD. GC B cells are defined as CD19, CD20, and CD38, IgD - B cells. Memory B cells, alternatively, express CD19, CD20, and CD27. Furthermore, human plasmablasts are defined as CD38, CD20, B cells that are in the process of downregulating surface BCR and most other surface Ags.

The GC is a highly active environment vital for proper functioning of the adaptive immune system. GC B cells undergo affinity maturation, which involves iterative cycles of clonal expansion, somatic hypermutation, and selection that result in class-switched memory B cells and Ab-secreting plasma cells (4, 5). How high-affinity B cells are selected in the GC is not entirely clear. Increased Ag capture and presentation lead to increased rates of cell division (5, 6). It is also possible that actively proliferating GC B cells produce unique signals that promote their survival and proliferation. Additionally, GC B cell signaling is regulated by protein tyrosine phosphatases (7, 8). For example, cell surface CD22 can recruit phosphatases, such as SHP-1, to attenuate BCR signaling (8, 9). Opposing this activity are NADPH oxidases, such as DUOX1, which produce $H_2O_2$ and lower BCR signaling thresholds by reversibly inhibiting phosphatases (2). The environment surrounding the GC simulates NADPH oxidase, which produces endogenous ROS (10). In turn, ROS oxidize the extracellular compartment and activate the BCR signaling pathway, creating a positive feedback loop. BCR signaling governs B cell functions, and activation and termination of BCR signaling are finely tuned by multiple levels of regulation in healthy cells.

Although the biochemistry of BCR signaling is well understood in model systems, little is known about the quality of in vivo BCR signaling in mature, healthy human B cells. Addressing this gap by mapping the influence of ROS on healthy B cell signaling is important for placing into context the extreme BCR signaling and $H_2O_2$ responses observed in B cell diseases and disorders (11). In this study, we used high-dimensional mass cytometry, phospho-specific flow cytometry, and novel computational data analysis...
tools (12–14) to better understand how ROS regulate BCR signaling within subsets of primary human tonsillar B cells.

**Materials and Methods**

**Human samples**

Tonsils were obtained from children undergoing routine tonsillectomies in accordance with the Declaration of Helsinki following protocols approved by Vanderbilt University Medical Center Institutional Review Board. Single-cell suspensions were prepared and stored in liquid nitrogen.

**Abs**

Fluorescent Abs for CD20, IgD, CD38, CD3, CD27, p-SRC, p-SYK, p-phospholipase C (PLCγ), and p-NF-κB were conjugated to BV421, PerCP-Cy5.5, FITC, PE-Cy7, BV405, BV550, PE, and Alexa Fluor 647 (BD Biosciences, Invitrogen, or BioLegend). Mass cytometry Abs are listed in Supplemental Table I.

**Fluorescent cytometry**

Aliquots of cryopreserved single-cell tonsillar samples were thawed into 10 ml warm media (RPMI 1640 [Mediatech, Manassas, VA] plus 10% FBS [Life Technologies, Grand Island, NY]), pelleted by centrifugation at 200 × g, washed with warm media, and pelleted again at 200 × g before resuspension in flow cytometry tubes. Resuspended samples rested for 15 min in a 5% CO2 incubator at 37°C. Cells were washed once in double distilled H2O, suspended in double distilled H2O, and collected by centrifugation at 200 × g, re-suspended in warm media (RPMI 1640 [Mediatech, Manassas, VA] plus 10% FBS [Life Technologies, Grand Island, NY]) for 5 min at room temperature, then stained with 250 nM iridium intercalator and anti-PE-IgG, IgM, IgA, and p–PLCγ (Fluidigm) for 30 min at room temperature. Cells were washed once in PBS, resuspended in double distilled H2O, and collected on a CyTOF 1.0 at the Vanderbilt Flow Cytometry Shared Resource. Cells stained with panel 2 (Supplemental Table I) were stained immediately after thawing, except for SHP-1, which was stained after permeabilization. Mass cytometry data files were evaluated using manual gating and viSNE (12) in Cytobank. Data were transformed using an arcsinh scale (cofactors of 15, except for SHP-1, which had a cofactor of 5). viSNE maps were generated using the following markers: SHP-1, CD40, IgD, CD3, CD3, CD19, CD20, CD86, CD22, CD44, CD38, CD27, CD79B, and HLA-DR.

**Results and Discussion**

A subset of human tonsillar B cells was initially observed to respond to a 2-min stimulation by 3.3 mM H2O2 by phosphorylating upstream members of the BCR signaling pathway, including SYK and Src family kinases (SKFs). This H2O2-sensitive population varied in abundance from 7.3 to 33.24% of CD3+ cells (Supplemental Fig. 1) and generally expressed higher levels of CD20 compared with other tonsillar B cells (Fig. 1). In previous reports, naive B cells in peripheral blood did not respond to 3.3 mM H2O2 (15). The H2O2 response of the CD20hi CD3− B cells distinguished these cells from other tonsillar cells and contrasted with the B cell response to other stimuli, such as CD40L, which showed no significant signaling differences across the full range of CD20 expression levels.

**Mass cytometry**

Single-cell tonsillar samples were thawed the same way as samples prepared for fluorescent cytometry. For mass cytometry panel 1, one tonsil sample was left unstimulated and one sample was stimulated with H2O2 (Fisher Scientific) for 2 min. Cells were fixed (parafomaldehyde) and stained for extracellular targets (Supplemental Table I). After methanol permeabilization, cells were stained for IgG, IgM, IgA, and p–PLCγ-PE in cell staining media for 15 min at room temperature, then stained with 250 nM iridium intercalator and anti-PE (Fluidigm) for 30 min at room temperature. Cells were washed once in PBS, resuspended in double distilled H2O, suspended in double distilled H2O, and collected on a CyTOF 1.0 at the Vanderbilt Flow Cytometry Shared Resource. Cells stained with panel 2 (Supplemental Table I) were stained immediately after thawing, except for SHP-1, which was stained after permeabilization. Mass cytometry data files were evaluated using manual gating and viSNE (12) in Cytobank. Data were transformed using an arcsinh scale (cofactors of 15, except for SHP-1, which had a cofactor of 5). viSNE maps were generated using the following markers: SHP-1, CD40, IgD, CD3, CD3, CD19, CD20, CD86, CD22, CD44, CD38, CD27, CD79B, and HLA-DR.

**FIGURE 1.** CD20hi B cells in human tonsil were sensitive to H2O2. (A) Contour plots show gating for CD3+ cells and CD3−CD20− T cells in human tonsil. (B) Contour plots show p-SYK and p-SFK in CD3+ tonsillar B cells left unstimulated or stimulated by 3.3 mM H2O2 for 2 min. Sensitivity to H2O2 in a CD20hi B cell population is indicated (gray arrows). Plots are representative of three tonsils.

**FIGURE 2.** Mass cytometry revealed H2O2 responder population as CD20hi, CD38−, and IgD−. (A) Contour plots show gating for CD19+ B cells and CD3− T cells in human tonsil. (B) Contour plots show p–PLCγ in CD19+ tonsil B cells left unstimulated or stimulated by 3.3 mM H2O2 for 4 min. Cells that were sensitive to H2O2 stimulation were labeled responder (R) cells and contrasted with nonresponder cells (NR). (C) A heat map shows the median fold change in CD3+ T cells, R, and NR subsets.
Thus, a novel H$_2$O$_2$ signaling response distinguished a CD20$^{hi}$ subset of tonsillar B cells. Comprehensive characterization of H$_2$O$_2$-responsive B cells by mass cytometry

To determine the identity of the H$_2$O$_2$-responsive cells, a high-dimensional mass cytometry panel designed to characterize mature B cells was developed (Supplemental Table I). The H$_2$O$_2$-sensitive cell population was gated and labeled as "responder" cells, and the signature of protein expression was contrasted with cells labeled as "nonresponders" or CD3$^+$ T cells (Fig. 2A, 2B). The H$_2$O$_2$-sensitive responder cells were characterized by a CD20$^{hi}$, CD3$^-$, IgD$^+$ phenotype that contrasted with the other evaluated populations of nonresponder cells and CD3$^+$ cells (Fig. 2C). Each point represents the average of three individual tonsil specimens ($n = 3$) stimulated for 2 min with the indicated concentration of H$_2$O$_2$, except for the 0.04 mM and 0.12 mM H$_2$O$_2$ stimulated conditions (where $n = 2$). Red squares represent GC B cells and blue circles represent naive B cells. Error bars denote the SD for each point.

GC B cells were hypersensitive to H$_2$O$_2$ stimulation

BCR signaling normally triggers a complex, interconnected network of effector signaling pathways (15), and it is currently not known how the quality, magnitude, and duration of BCR signaling "programs" a B cell for contrasting functional outcomes ranging from cell death to proliferation. Phospho-proteins in the BCR signaling network that are rapidly phosphorylated following H$_2$O$_2$ stimulation might act as effectors of secondary messenger signaling. To identify H$_2$O$_2$ signaling effectors and better delineate the H$_2$O$_2$ sensitivity of B cell populations, a fluorescent panel was developed and cells from three human tonsils were stimulated with varying doses of H$_2$O$_2$ for 2 min (Fig. 3). Naive, GC, memory, and plasmablast B cell subsets were distinguished using canonical markers CD3, CD20, CD38, CD27, and IgD (Fig. 3A). Observed B cell subsets responded to H$_2$O$_2$ in a dose-dependent manner seen through the phosphorylation of SFK, PLC$_Y$, and SYK; however, GC B cells were the most sensitive to H$_2$O$_2$ at all concentrations (Fig. 3B, 3C).

H$_2$O$_2$ sensitivity may be an intrinsic characteristic of GC B cells that is necessary for BCR regulation within an active GC. GC B cells may use endogenously produced H$_2$O$_2$ as a modulator of BCR signaling, whereas BCRs undergo iterative modification. In fact, loss of BCR signaling in healthy B cells reduces B cell survival, and sustained BCR signaling capability...
is essential for B cell development and survival (17). Observed H2O2 hypersensitivity of GC B cells (Fig. 3) may be an important feature of accelerating the GC reaction; alternatively, this redox sensitivity may help to cull B cells that do not appropriately execute the delicate process of somatic hypermutation. These results help to place in context the observation that lymphoma B cells are especially sensitive to ROS (18). Prior studies revealed that lymphoma B cells undergo rapid, ROS-mediated apoptosis when glutathione is depleted and that stimulation of lymphoma B cells using anti-BCR F(ab′)2 and H2O2 negates suppression of BCR signaling that distinguishes clinically relevant lymphoma negative prognostic cells in follicular lymphoma (11).

Heterogeneous SHP-1 expression across B cell populations

Previous data from GCs generated within transgenic mice reported that GC B cells do not robustly respond to Ag or anti-IgM stimulation compared with non-GC B cells due to colocalization of SHP-1 with the BCR (8). To study this relationship in humans, a single-cell approach was used to measure total SHP-1 levels within human tonsillar B cell subsets and quantify any correlation between total SHP-1 protein expression and B cell population identity. Furthermore, an unsupervised computational approach was used to characterize GC B cells and determine whether additional heterogeneity might exist within this or other B cell populations (12, 13). Elevated phosphate levels of GC B cells compared with other B cell subsets might explain why GC B cells were hypersensitive to H2O2 stimulation. To evaluate this hypothesis, an Ab for SHP-1 was added to the mass cytometry panels (Supplemental Table I). B cell subsets were identified by viSNE analysis using the same key markers as in fluorescent experiments (Fig. 4). viSNE revealed heterogeneous expression of SHP-1 within naive, GC, and memory B cell populations. Each of these B cell populations contained both high and low SHP-1–expressing cells. In contrast, plasmablasts expressed a consistent, low level of SHP-1. SHP-1 expression contrasted strongly with canonical subset marker expression patterns, which were enriched in subset-specific ways, such as CD20 and CD38 (Fig. 4B). In the present study, SHP-1 expression was uncorrelated with H2O2 sensitivity across the B cell stages. Plasmablasts and naive B cells expressed contrasting levels of SHP-1 and had comparable H2O2 sensitivity, whereas GC and naive B cells had contrasting H2O2 sensitivity despite similar median levels and per-cell distributions of SHP-1 expression (Figs. 3, 4).

Because SHP-1 expression did not correlate with B cell subset, it is possible that the observed heterogeneity of SHP-1 expression is due to transient differences within B cell subsets that are not reflective of stage, but rather recent stimulation experience. A recent study demonstrated that a subpopulation of light zone GC B cells had more robust BCR signaling compared with all GC B cells (19). Our study was not powered to look at light zone/dark zone differences, but the data suggested that light zone GC B cells may be the GC B cells that are higher for SHP-1. Within the GC B cell subset, the cells on the viSNE map that expressed higher levels of SHP-1 also expressed higher levels of CD40, HLA-DR, CD22, and CD86 (Fig. 4A). These proteins relate to T cell signaling interactions and suggest a shift in the signaling relationship between T follicular helper cells and GC B cells.

These results provide new information regarding redox-sensitive signaling in B cell networks that may act to control the outcomes of GC reactions. Precisely how ROS regulate BCR signaling within GGs remains to be seen; however, the findings in the present study indicate that redox cues specifically impact human GC B cell signaling. These results revealed unknown human GC B cell signaling responses to ROS that can be used as a reference point for studies of diseases originating in cells with GC characteristics, such as B cell lymphomas.

Disclosures

J.M.I. is a cofounder and board member of Cytobank, Inc. The other authors have no financial conflicts of interest.

References

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Marker Type Key:
α - Mass cytometry panel 1 only
β - Mass cytometry panel 2 only
δ - Both panels

All antibodies were purchased from Fluidigm, unless otherwise specified
*BD Biosciences antibody
**Cell signaling technology antibody

Table S1. Mass Cytometry Panels.
Supplemental Figure 1. Populations (CD3$^+$ cells and CD3$^-$CD20$^+$ cells) defined in figure 1 for A-C and gating schematic for D and E (Naïve B cells, GC B cells, Memory B cells, plasmablasts) shown in figure 3. (A) Contour plots show p-NFκB in unstimulated cells and cells stimulated by CD40L for 15 minutes in CD3$^+$ cells. (B-D) Cells were either left unstimulated or stimulated by 3.3 mM of H$_2$O$_2$ for 2 minutes. (B) Contour plots show p-SFK (i.e. p-LCK) in CD20$^-CD3^+$ tonsillar T cells (C) Contour plots show two other CD3$^+$ healthy tonsil specimens (T13 left-two plots, V015T right-two plots) and their response to H$_2$O$_2$ stimulation. Sensitivity to H$_2$O$_2$ in a CD20$^+$ B cell population is indicated (gray arrows). (D) Population portions of identified B cell subsets for two tonsils is shown. (E) Contour plots show raw data for B cell population responses to H$_2$O$_2$ (bottom row). Data for one representative tonsil (T13, n=3) is shown.