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Cutting Edge: Redox signaling hypersensitivity distinguishes human germinal center B cells

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Abstract

Differences in the quality of B-cell antigen receptor (BCR) signaling control key steps of B cell maturation and differentiation. Endogenously produced H₂O₂ is thought to fine tune the level of BCR signaling by reversibly inhibiting phosphatases. However, relatively little is known about how B cells at different stages sense and respond to such redox cues. Here, we used phospho-specific flow cytometry and high-dimensional mass cytometry (CyTOF) to compare BCR signaling responses in mature human tonsillar B cells undergoing germinal center (GC) reactions. GC B cells, in contrast to mature naïve B cells, memory B cells, and plasmablasts, were hypersensitive to a range of H₂O₂ concentrations and responded by phosphorylating SYK and other membrane proximal BCR effectors in the absence of BCR engagement. These findings reveal that stage specific redox responses distinguish human GC B cells.

INTRODUCTION

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 B-cell antigen receptor (BCR) signaling, secondary messengers control the signaling context and help determine functional outcomes in B cells. H₂O₂ is the primary reactive oxygen species (ROS) produced by B cells. H₂O₂ amplifies BCR signaling by transiently inhibiting BCR-associated protein tyrosine phosphatases (PTPs) (1). H₂O₂ is also produced as part of innate immune responses to wounds and infection (2). However, it is not known what impact H₂O₂

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has on healthy human B-cell signaling responses and whether B cells undergoing GC reactions respond differently to H₂O₂.

Seconds after BCR crosslinking, a network of signaling molecules becomes activated through post-translational modifications. As signaling directs B cells down differentiation pathways, B cells adopt well characterized signatures defined primarily by protein expression (3). Naïve B cells in humans are defined by expression of CD19, CD20, and IgD. GC B cells are defined as CD19⁺, CD20^{hi}, CD38⁺, IgD⁻ B cells. Memory B cells, on the other hand, express CD19, CD20, and CD27. Furthermore, human plasmablasts are defined as CD38^{hi}, CD20^{lo} cells that are in the process of down regulating surface BCR and most other surface antigens.

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 antibody-secreting plasma cells (4, 5). How high-affinity B cells are selected in the GC is not entirely clear. Increased antigen capture and presentation leads 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. In addition, GC B cell signaling is regulated by PTPs (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 (NOXs), such as DUOX1, which produce H₂O₂ and lower BCR signaling thresholds by reversibly inhibiting phosphatases (2). The environment surrounding the BCR simulates NOX, 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 is finely tuned by multiple levels of regulation in healthy cells.

While 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 H₂O₂ responses observed in B-cell diseases and disorders (11). Here, 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 & 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 (VUMC) Institutional Review Board. Single cell suspensions were prepared and stored in liquid nitrogen.

ANTIBODIES

Fluorescent antibodies for CD20, IgD, CD38, CD3, CD27, p-SRC, p-SYK, p-PLC γ , and p-NF κ B were conjugated to BV421, PerCP Cy5.5, FITC, PE-Cy7, BUV395, BV570, BV605, PE, and AlexaFluor647 (BD Biosciences, Invitrogen, or Biolegend). Mass cytometry antibodies are listed in Supplemental Information (Supplemental Table 1).

FLUORESCENT CYTOMETRY

Aliquots of cryopreserved single cell tonsillar samples were thawed into 10 mL of warm media (RPMI 1640 (Mediatech, Inc., Manassas, VA) + 10% FBS (Gibco[®], life technologies, Grand Island, NY)), pelleted by centrifugation at $200 \times g$, washed with warm media and pelleted again at $200 \times g$ before re-suspension in flow cytometry tubes. Re-suspended samples rested for 15 minutes in a 5% CO₂ incubator at 37 °C. Each rested sample was either left unstimulated or stimulated with H₂O₂ (Fisher Scientific, Fair Lawn, NJ) for 2 minutes or CD40L plus enhancer (Enzo Life Sciences, Inc., Farmingdale, NY) for 15 minutes. CD40L and enhancer were prepared per manufacturer's recommendation. Cells were fixed with 1.6% paraformaldehyde (PFA, Electron Microscopy Services, Fort Washington, PA) for 5 minutes at room temperature (RT) following stimulation, washed with PBS (HyClone Laboratories, Logan, UT), pelleted at $800 \times g$, and permeabilized by 100% ice cold methanol (MeOH) (Fisher Scientific) in a -20 °C freezer overnight. Cells were washed once with PBS and once with cell staining media (CSM) composed of PBS + 1% bovine serum albumin (BSA, Fisher Scientific). For each condition, 1×10^6 tonsillar cells were stained in 100 μ L of CSM. Samples were analyzed using a five-laser BD LSR II (Becton Dickinson, Franklin Lakes, New Jersey) at the Vanderbilt Flow Cytometry Shared Resource (VFCSR) and evaluated using Cytobank software.

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 minutes. Cells were fixed (PFA) and stained for extracellular targets (Supplemental Table 1). After MeOH permeabilization, cells were stained for IgG, IgM, IgA, and p-PLC γ -PE in CSM for 15 minutes at RT, then stained with 250 nM iridium intercalator and anti-PE (Fluidigm) for 30 minutes at RT. Cells were washed once in PBS, once in ddH₂O, suspended in ddH₂O and collected on a CyTOF 1.0 at the VFCSR. Cells stained with panel 2 (Supplemental Table 1) 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, HLA-DR.

RESULTS AND DISCUSSION

A subset of B cells responded robustly to H₂O₂ stimulation

A subset of human tonsillar B cells was initially observed to respond to a 2 minute stimulation by 3.3 mM H₂O₂ by phosphorylating upstream members of the BCR signaling pathway, including SYK and Src family kinases (SFKs). This H₂O₂-sensitive population varied in abundance from 7.3% to 33.24% of CD3⁻ cells (Supplementary Fig. 1) and generally expressed higher levels of CD20 compared to other tonsillar B cells (Fig. 1). In previous reports, naïve B cells in peripheral blood did not respond to 3.3 mM H₂O₂ (15). The H₂O₂ response of the CD20^{hi} 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 (Supplementary Fig. 1). Thus, a novel H₂O₂ signaling response distinguished a CD20^{hi} subset of tonsillar B cells.

Comprehensive characterization of H₂O₂-responsive B cells by mass cytometry

To determine the identity of the H₂O₂-responsive cells, a high-dimensional mass cytometry panel designed to characterize mature B cells was developed (Supplemental Table 1). The H₂O₂-sensitive cell population was gated and labeled as “responder” cells (R) and the signature of protein expression was contrasted with cells labeled as “non-responders” (NR) or CD3⁺ T cells (Fig. 2A, B). The H₂O₂-sensitive responder cells were characterized by a CD20^{hi}, CD38⁺, IgD⁻ phenotype that contrasted with the other evaluated populations of non-responder cells and CD3⁺ cells (Fig. 2C). This observed responder cell phenotype suggested a GC B cell identity (3, 16). In agreement with this, a strong relation was seen between the fraction of H₂O₂-sensitive responding cells and the abundance of GC B cells in each tonsil (Supplementary Fig. 1).

Germinal center B cells were hypersensitive to H₂O₂ 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 H₂O₂ stimulation might act as effectors of secondary messenger signaling. To identify H₂O₂ signaling effectors and better delineate the H₂O₂ sensitivity of B cell populations, a fluorescent panel was developed and cells from three human tonsils were stimulated with varying doses of H₂O₂ for 2 minutes (Fig. 3). Naïve, 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 H₂O₂ in a dose dependent manner seen through the phosphorylation of SFK, PLC γ , and SYK; however, GC B cells were the most sensitive to H₂O₂ at all concentrations (Fig. 3B,C).

H₂O₂ 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 H₂O₂ as a modulator of BCR signaling, while B-cell receptors 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 α -BCR F(ab')₂ and H₂O₂ negates suppression of BCR signaling that distinguishes clinically relevant lymphoma negative prognostic (LNP) cells in follicular lymphoma (19).

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 antigen or anti-IgM stimulation compared to 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 phosphatase levels of GC B cells compared to other B cell subsets might explain why GC B cells were hypersensitive to H₂O₂ stimulation. To evaluate this hypothesis, an antibody for SHP-1 was added to the mass cytometry panels (Supplemental Table 1). 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 naïve, 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). SHP-1 expression was uncorrelated with H₂O₂ sensitivity across the B cell stages studied here. Plasmablasts and naïve B cells expressed contrasting levels of SHP-1 and had comparable H₂O₂ sensitivity, whereas GC and naïve B cells had contrasting H₂O₂ sensitivity despite similar median levels and per-cell distributions of SHP-1 expression (Fig. 3 and 4).

Since 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 to all GC B cells (20). 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 (T^{FH}) 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 GCs remains to be seen; however, the findings here 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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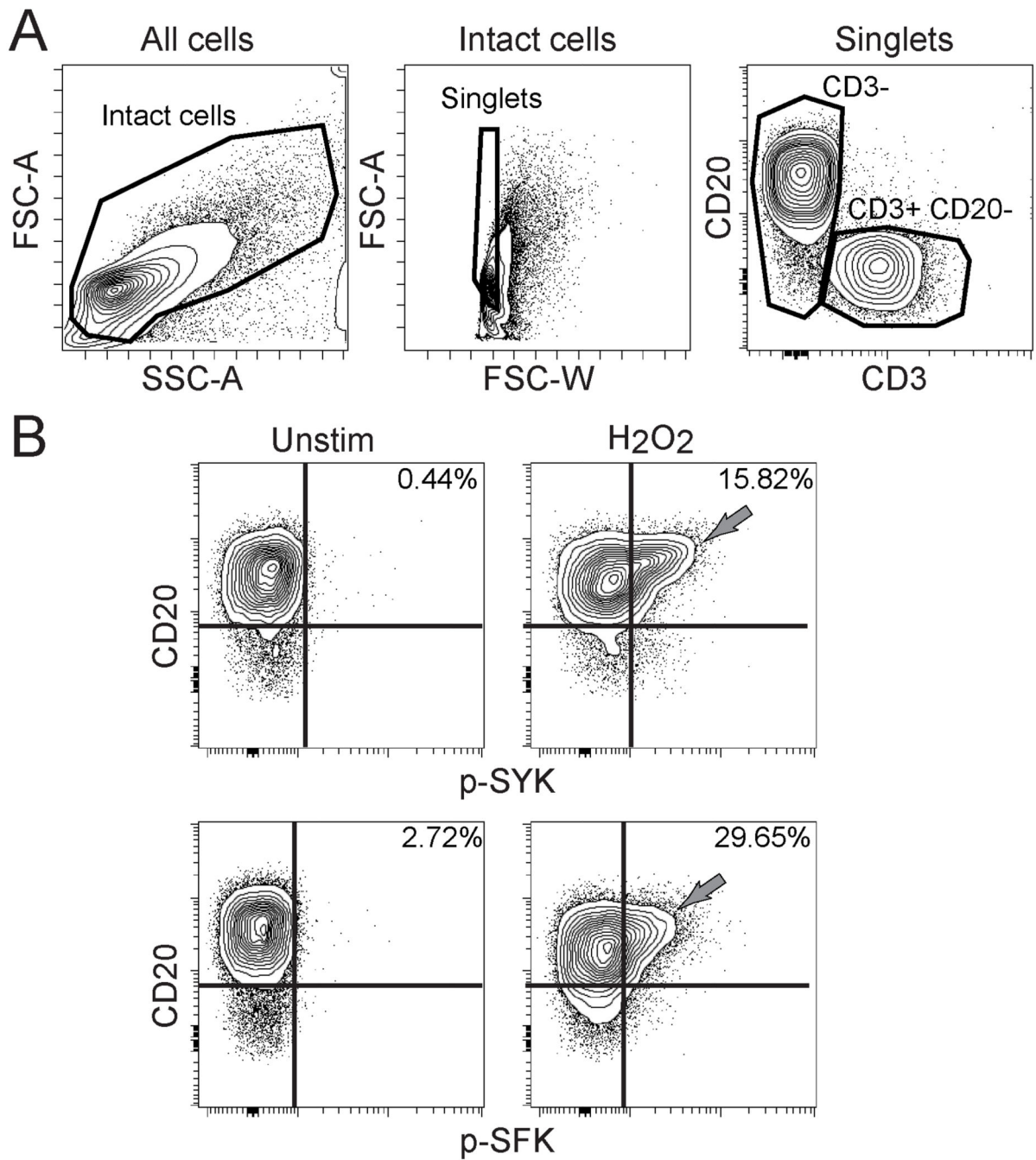


Figure 1. CD20^{hi} B cells in human tonsil were sensitive to H₂O₂
 (A) Contour plots show gating for CD3⁻ cells and CD3⁺ CD20⁻ T cells in human tonsil. (B) Contour plots show phosphorylated SYK (p-SYK) and p-SFK in CD3⁻ tonsillar B cells left unstimulated or stimulated by 3.3 mM H₂O₂ for 2 minutes.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

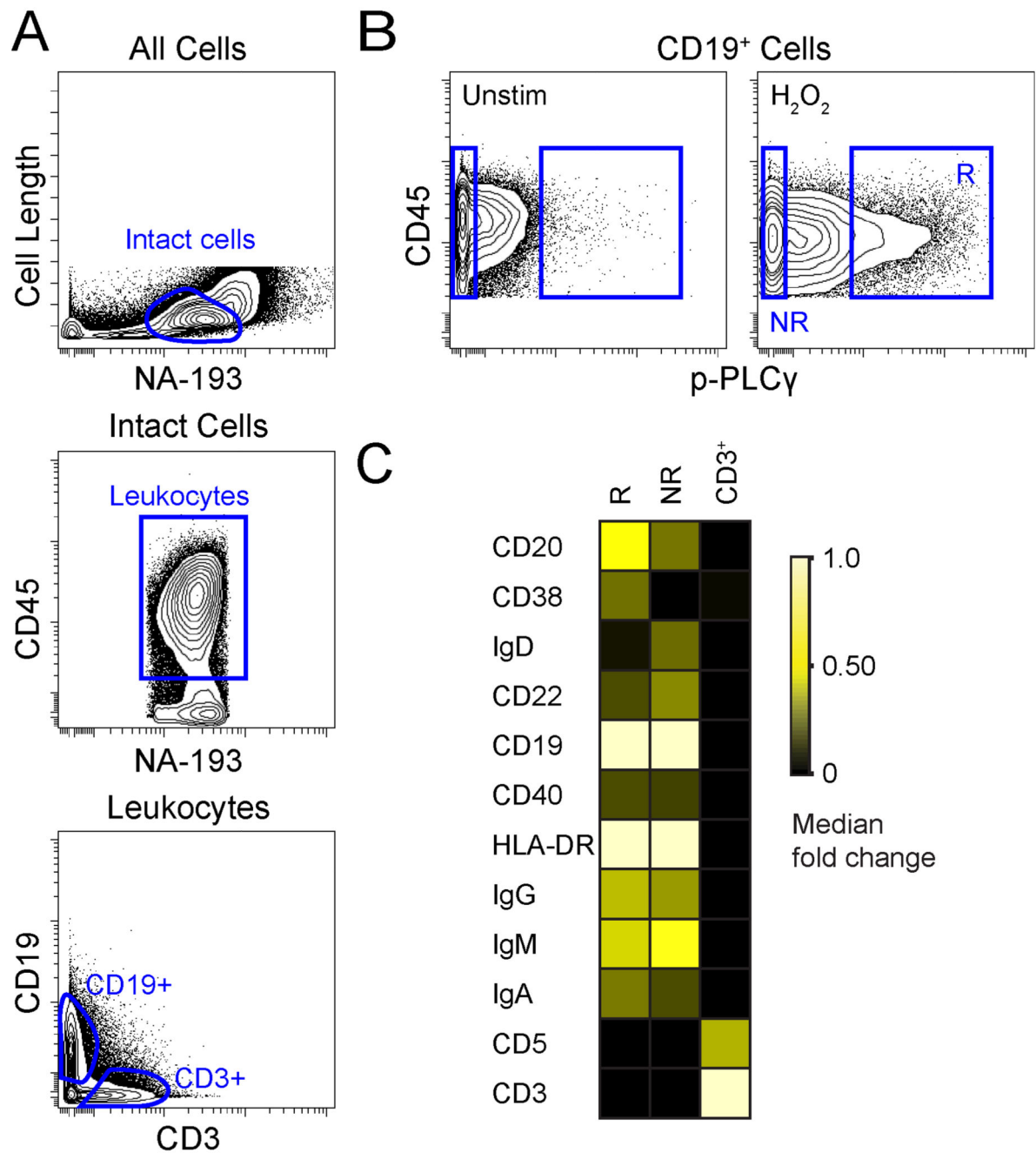


Figure 2. Mass Cytometry Revealed H₂O₂ ‘Responder’ Population as CD20^{hi}, CD38⁺, and IgD⁻
 (A) Contour plots show gating for CD19⁺ B cells and CD3⁺ T cells in human tonsil. (B) Contour plots show p-PLC γ in CD19⁺ tonsil B cells left unstimulated or stimulated by 3.3 mM H₂O₂ for 4 minutes. Cells that were sensitive to H₂O₂ stimulation were labeled responder (R) cells and contrasted with non-responder cells (NR). (C) Heat map shows the median fold change CD20 and CD38 in CD3⁺ T cells, R, and NR subsets.

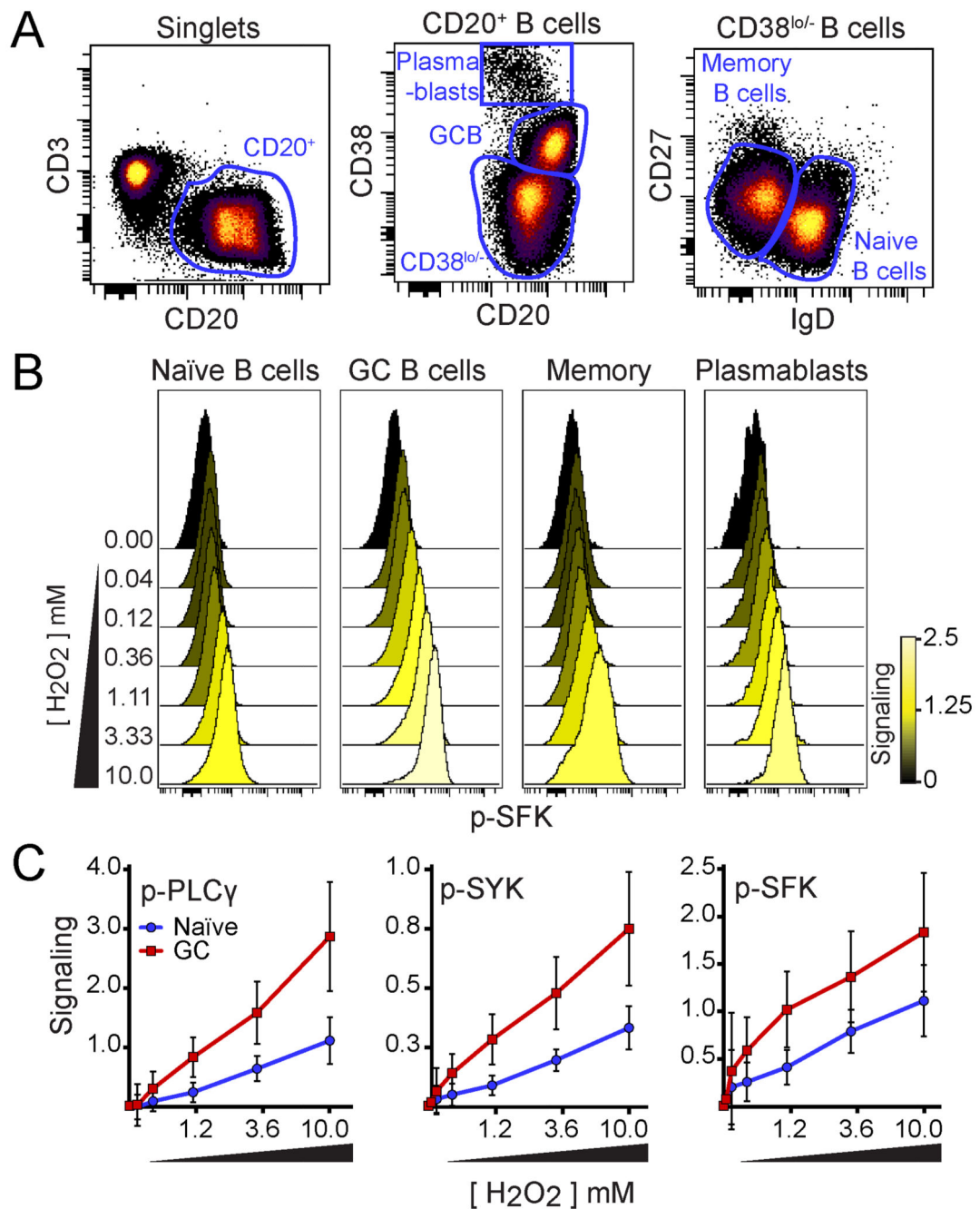


Figure 3. GC B cells were hypersensitive to H₂O₂

(A) Density dot plots show gating for identification of plasmablasts, GC B cells, memory B cells, and naïve B cells in human tonsils. (B) Histogram overlays show p-SFK in each B cell population (shown in A) following 2 minutes of 3.3 mM of H₂O₂ (n=3, representative data shown). Color denotes median fold change in p-SFK expression compared to unstimulated (0 mM of H₂O₂). (C) Plots illustrate the median fold change in p-PLC γ , p-SYK, and p-SRC in H₂O₂-stimulated conditions compared to the unstimulated condition (arcsinh scale). Each point represents the average of three individual tonsil specimens (n=3) stimulated for 2

minutes with the indicated concentration of H₂O₂, except for the 0.04 mM and 0.12 mM H₂O₂ stimulated conditions (where n=2). Red squares represent GC B cells and blue circles represent naïve B cells. Error bars denote the standard deviation for each point.

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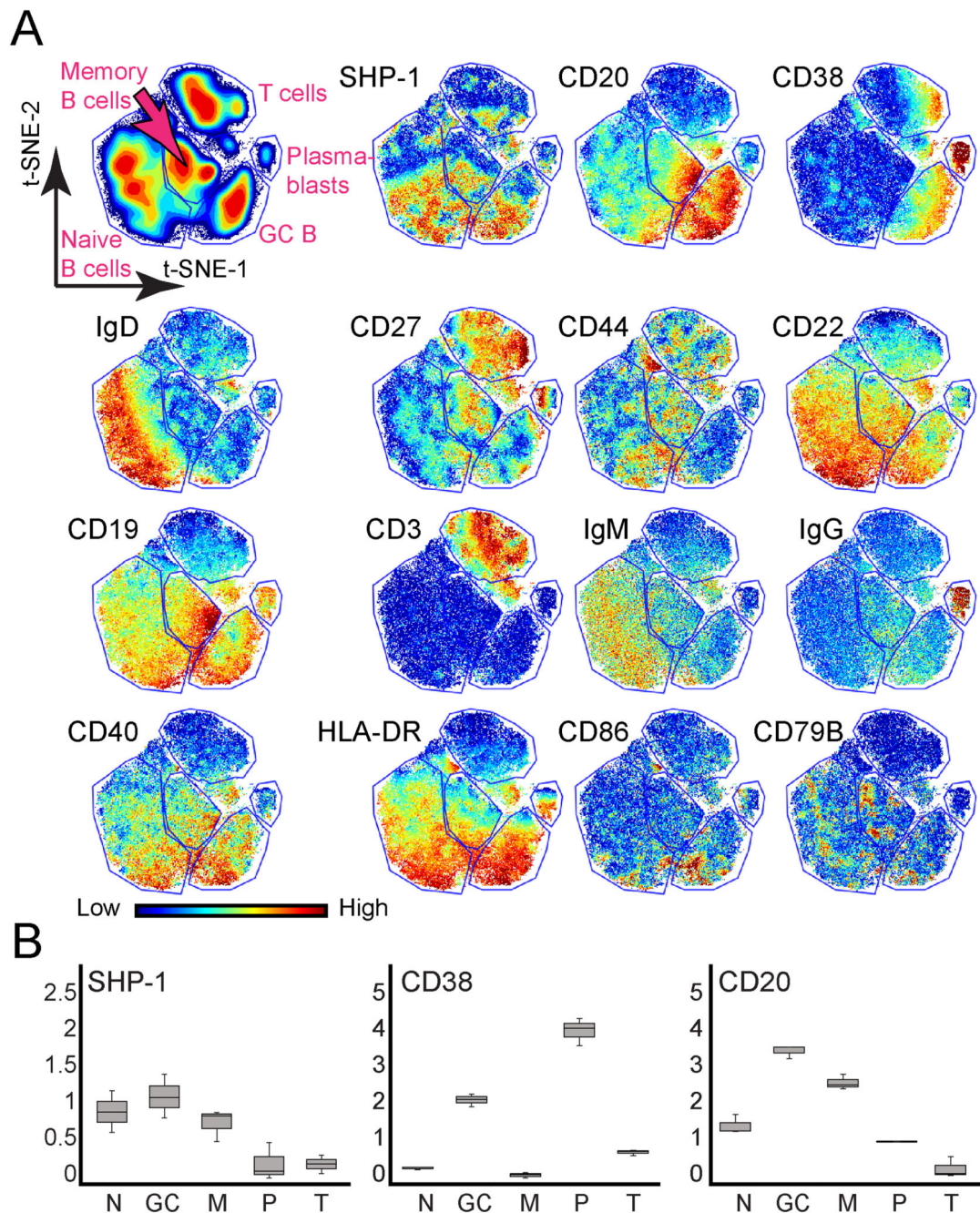


Figure 4. SHP-1 expression was heterogeneous within B cell populations

viSNE maps show $CD45^+$ leukocytes arranged based on marker expression profiles (see gating in Fig. 2A). Color denotes protein expression, as indicated. (A) Gates were drawn around the main populations identified by viSNE, using protein expression to identify each population. $CD19^+$ B cells were subdivided into naïve B cells ($CD38^-CD27^-IgD^+$), GC B cells ($CD20^{hi}CD38^+$), memory B cells ($CD38^-CD27^+IgD^-$), and plasmablasts ($CD20^-CD38^{hi}$) and compared to $CD3^+$ T cells. One representative tonsil of four analyzed is shown. (B) Box and Whisker plots illustrate expression of SHP-1, CD38, and CD20 proteins

across three tonsil specimens for naïve B cells, GC=germinal center B cells, M=memory B cells, P=plasmablasts, and T= T cells. Median of each marker is indicated by a black line. Bars denote the minimum and maximum observed MFI of each marker.

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