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Prolonged Production of Reactive Oxygen Species in Response to B Cell Receptor Stimulation Promotes B Cell Activation and Proliferation

Matthew L. Wheeler*† and Anthony L. DeFranco*

We have investigated the intracellular sources and physiological function of reactive oxygen species (ROS) produced in primary B cells in response to BCR stimulation. BCR stimulation of primary resting murine B cells induced the rapid production of ROS that occurred within minutes and was maintained for at least 24 h after receptor stimulation. While the early production of ROS (0–2 h) was dependent on the Nox2 isoform of NADPH oxidase, at later stages of B cell activation (6–24 h) ROS were generated by a second pathway, which appeared to be dependent on mitochondrial respiration. B cells from mice deficient in the Nox2 NADPH oxidase complex lacked detectable early production of extracellular and intracellular ROS after BCR stimulation but had normal proximal BCR signaling and BCR-induced activation and proliferation in vitro and mounted normal or somewhat elevated Ab responses in vivo. In contrast, neutralizing both pathways of BCR-derived ROS with the scavenger N-acetylcysteine resulted in impaired in vitro BCR-induced activation and proliferation and attenuated BCR signaling through the PI3K pathway at later times. These results indicate that the production of ROS downstream of the BCR is derived from at least two distinct cellular sources and plays a critical role at the later stages of B cell activation by promoting sustained BCR signaling via the PI3K pathway, which is needed for effective B cell responses to Ag.

Engagement of the BCR initiates a series of tightly controlled signaling events that in self-reactive B cells promote tolerance and in B cells responding to foreign Ag promote activation (1). Signaling downstream of the BCR is initiated by the Src family protein tyrosine kinases Lyn, Fyn, and Blk, which phosphorylate targets to induce activation of a number of signaling pathways required for subsequent B cell responses (2, 3). Among the targets of these kinases are the BCR Ig-α and Ig-β subunits, and their phosphorylation recruits Syk, representing an amplification loop that is countered by several feedback inhibitory pathways (2, 4). An especially well characterized feedback inhibitory pathway involves activation and recruitment, in proximity to the BCR, of the protein tyrosine phosphatase (PTPase) Src homology region 2 domain-containing phosphatase-1 (SHP-1), which broadly inhibits signaling by dephosphorylating proximal BCR signaling intermediates such as Syk (5, 6). Feedback inhibition of BCR signaling can also target specific pathways downstream of the BCR, as is the case with the lipid phosphatases PTEN and SHIP, which negatively regulate signaling through the PI3K pathway (7). The balance between the activities of BCR-stimulated protein kinases and the opposing phosphatases is likely critical for controlling the ultimate outcome of Ag encounter by the B cell.

Reactive oxygen species (ROS) have long been known to be potent inhibitors of PTPases and the lipid phosphatase PTEN through their ability to oxidize catalytic-site cysteines in these enzymes to render them transiently inactive (8–11). Although NADPH oxidase-derived ROS production by phagocytic cells is well appreciated for its role in anti-microbial defense, non-phagocytic cells, including lymphocytes, are also known to express functional machinery of the NADPH oxidase complex and can generate ROS in response to activating signals such as Ag receptor engagement (12–14). Activated cells also generate ROS as a by-product of normal mitochondrial respiration (15). Thus, it has been hypothesized that low-level production of ROS serves as a mechanism to inhibit phosphatase activity and thereby promote BCR signal amplification (16). Interesting in this regard, growth factor receptor signaling, which has many parallels to Ag receptor signaling, has been shown to be amplified by low-level ROS generation (17, 18).

Several studies have investigated whether low-level production of ROS downstream of the BCR or TCR can influence the magnitude of signaling or cell activation in response to cognate Ag stimulation (12, 19–22); however, the results have been inconclusive and were focused mainly on the signaling function of ROS generated early after Ag receptor engagement. In this study, we sought to examine the role of ROS production in regulating BCR signaling both at early times upon initial stimulation and later during the activation of mature B cells. We found that ROS production in response to BCR stimulation arises from two distinct sources: one that is dependent on the Nox2 isoform of NADPH oxidase and occurs within minutes of BCR cross-linking, and a second that occurs at later times after BCR stimulation and is likely derived from mitochondrial sources. Importantly, we found that B cells deficient in early Nox2-dependent ROS production had no major defects in proximal BCR signaling or B cell activation and mounted normal or enhanced Ab responses to a T cell.

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Abbreviations used in this article: GC, germinal center; HEL, hen egg lysozyme; NAC, N-acetylcysteine; NP-CGG, 4-hydroxy-3-nitrophenylacetyl-haptenated chicken gamma globulin; ODN, oligodeoxynucleotide; PTPase, protein tyrosine phosphatase; ROS, reactive oxygen species; SHIP-1, Src homology region 2 domain-containing phosphatase-1; WT, wild-type.
dependent Ag. In contrast, neutralizing ROS produced at later times attenuated BCR-dependent sustained PI3K signaling and ultimately resulted in defective activation and proliferation in response to BCR stimulation. These results show that whereas early NADPH oxidase-derived ROS are dispensable for BCR signal amplification in naive resting B cells, the continuous production of ROS at later times during the activation process is critical for maintaining some components of BCR signal transduction and for optimal Ag-induced B cell activation and cell checkpoint activity.

Materials and Methods

Mice

Mice were between the ages of 7 and 12 wk for most experiments. B6 mice (000664; C57BL/6) and Ncf1 mutant mice (004742; C57BL/6-Ncf1m1J) (23) were purchased from The Jackson Laboratory. MD4 transgenic (B220-2) mice were obtained from J. Cyster (University of California, San Francisco) and crossed to the Ncf1m1J background for some experiments. All animals were housed in a specific pathogen-free facility at the University of California, San Francisco according to university and National Institutes of Health guidelines. Animal use was approved by the Institutional Animal Care and Use Committee.

Abs, flow cytometry analysis, and B cell purification

Fluorophore-conjugated Abs directed against the following molecules were used: B220 (RA3-6B2), CD23 (B34), CD68 (G11), CD69 (H1.2F3), CD95 (Fas), GL7 (Ly77), IgD (11-26.2-c), and CD19 (I3D3) all from BD Pharmingen; CD24 (M1/69) from BioLegend; CD93 (AA4.1) from eBioscience, IgM (goat polyclonal F(ab) monomer, μ-chain specific) from Jackson Immunoresearch. Cells were analyzed on an LSR II (BD Pharmingen). B cell populations in the spleen and bone marrow were stained for 30 min with anti-B220–PE–Cy7, CD93–allophycocyanin, CD23–PE, and IgM–FITC F(ab) monomer, and mature and immature B cell subpopulations were distinguished using the Almman protocol (24). Dead cells were excluded by propidium iodide (BioChemika) uptake. Purified B cells were isolated from spleens of 7- to 12-wk-old mice by negative selection using CD43 microbeads (Miltenyi Biotech), according to the manufacturer’s instructions, and passage through MACS LS separation columns (Miltenyi Biotech). All FACS data were analyzed with FlowJo version 9.3.3 (Tree Star).

Detection of ROS

Cytochrome c reduction to measure extracellular ROS production was performed as described (25). Briefly, purified splenic B cells were resuspended in 1 ml of HBSS with Ca/Mg salts plus 1% BSA at 5 × 10^6 cells/ml equilibrated at 37°C for 10 min. Cells (100 μl) were aliquoted into wells of Immulon-4 96-well plates (Thermo Scientific) containing cytochrome c (Sigma-Aldrich) (0.1 mM final concentration). Immediately after addition of stimulus (50 μg/ml anti-IgM F(ab)2, 500 μM PMA, or 500 ng/ml hen egg lysozyme for MD4 B cells), OD recordings at 550 nm and 490 nm were read at 2-min intervals in a 96-well kinetic plate reader (Spectra MAX plus; Molecular Devices). Relative superoxide production was derived from change in OD at 550–490 nm.

Chloriminescent detection of superoxide was performed as described (21) using Diogenes (National Diagnostics). Briefly, purified B cells were stimulated as described earlier except in the presence of Diogenes, and luminiscence was recorded at 5-min intervals on a luminoimeter (Spectra MAX M5; Molecular Devices).

BCR-localized ROS production was measured as described (19) by flow cytometry by stimulating cells with 20 μg/ml anti-IgM (Fab'2)2 conjugated to OxyBURST Green H2DCFDA, succinimidyl ester (Invitrogen) in pHol red-free HBSS with Ca/Mg salts plus 10 mM HEPES plus 1% FBS. ROS production was recorded at 488 nm at 5-min intervals by flow cytometry using an LSR II and gating B220+CD93 spleenic B cells or B220- non-B cells as a control.

Intracellular superoxide was recorded on total spleen cells loaded intracellularly with 5 μM MitoSOX Red (Invitrogen) for 20 min at 37°C and stained for surface B220 and CD93 as described earlier. For short-term recordings, B cells were stimulated at 37°C with 20 μg/ml anti-IgM F(ab)2 and MitoSOX Red fluorescence was recorded at 561 nm at 5-min intervals over 30 min by flow cytometry using an LSR II, and gating on mature B cells. B cell subpopulations were stained with anti-B220 and CD93, stained with 10 μg/ml anti-IgM F(ab)2 in complete Iscove’s medium containing 10% FBS for 2, 6, 12, and 24 h, and loaded with 5 μM MitoSOX Red for 30 min at 37°C prior to harvesting the cells at each time point. Cells were then washed and analyzed by flow cytometry as described to measure MitoSOX Red fluorescence. In some cases, cells were preincubated with 5 mM N-acetylcysteine (Sigma) for 30 min prior to stimulation or treated for the last 2 h of stimulation with 20 μg/ml antimycin-A (Sigma).

Analysis of calcium mobilization

Measurement of intracellular-free calcium levels was performed as described (26). Briefly, splenocytes were loaded with Indo-1-AM (Molecular Probes) (for p-ERK and p-AKT) or stimulated as described earlier except in the presence of Diogenes, and (21) using Diogenes (National Diagnostics). Briefly, purified B cells were loaded with Indo-1-AM (Molecular Probes) (for p-ERK and p-AKT) and B220, CD24 (in place of CD93), and CD23. Cells were analyzed by flow cytometry, and the phosphorylation status of each signaling molecule was analyzed within the mature follicular B cell population (B220+, CD24+, CD23+, IgMiso-). For analysis of late time points, purified splenic B cells were stimulated for 12 h with 10 μg/ml anti-IgM F(ab)2, cells were harvested and stimulated using anti-p-ERK1/2 or anti-p-S6 (Cell Signaling Technology) rabbit mAbs. In some cases, stimulation was carried out in the presence of 5 mM NAC (added prior to stimulation or for the last 2 h of stimulation) or 1 μM of the class I PI3K inhibitor GDC-0941 (Chemeida) added for the last 2 h of stimulation.

B cell activation and proliferation

For in vitro B cell activation analysis, purified splenic B cells were resuspended at 1 × 10^6 cells/ml in complete Iscove’s medium containing 10% FBS, in the presence or absence of 5 mM NAC, and equilibrated at 37°C for 30 min prior to stimulation. After stimulation with anti-IgM F(ab)2, cells were harvested at indicated times and stained with fluorescent Abs against surface CD69 (6–7 h) or CD86 (18 h). For in vitro proliferation, purified B cells, in the presence or absence of 5 mM NAC, were stimulated as described earlier with 10 μg/ml anti-IgM F(ab)2, 500 ng/ml CpG (Integrated DNA Technologies), or 10 μg/ml anti-Cd40 (BD Pharmingen) plus 10 ng/ml IL-4 (Roche). BrdU (BD Pharmingen) was added to cells (final concentration 10 μM) 36 h after stimulation, and cells were harvested for analysis 48 h after stimulation. Proliferation was assessed by incorporation of BrdU into B220+ B cells by intracellular staining using a BrdU flow kit (BD Pharmingen) following the manufacturer’s instructions. Cells were analyzed by flow cytometry using an LSR II and proliferation was derived from the percentage or absolute number of BrdU+ B cells.

Immunizations, germinal center cell analysis, ELISA, and ELISPOT assay

To assess T cell-dependent Ab production, mice were injected i.p. with 50 μg 4-hydroxy-3-nitrophenylacetyle-4-haptenated chicken gamma globulin (NP-CGG) precipitated in alum. Sera were collected on days 7 and 14 after immunization, and NP-specific IgM and IgG1 titers were measured by ELISA. For ELISA, 96-well plates (BD Falcon) were coated overnight with NPss-BSA and blocked with 2% FBS in 1× PBS (1–2 h), followed by incubation with serial dilutions of serum for 2 h at room temperature. NP-specific IgM and IgG1 were detected with HRP-conjugated anti-mouse
IgM and IgG1 Abs (Southern Biotech) and developed with 3,3',5,5'-tetramethylbenzidine substrate (Vector Laboratories). OD (450–570 nm) measurements were recorded with a 96-well microplate plate reader (VERSA max; Molecular Devices). Serum Ab titers were derived from the slope of the titration curve and normalized to a standard serum sample from a hyperimmunized mouse. Germinal center (GC) cell development was measured on days 7 and 14 from NP-CGG immunized mice by staining total spleen cells with Abs against B220, CD4, IgD, CD95 (Fas), and GL7 and defining GC B cells as B220+, IgDlo, Fas+, and GL7+. Numbers of NP-specific plasma cells were determined by ELISPOT assay. Briefly, splenocytes from NP-CGG immunized mice were resuspended in complete Iscove’s medium containing 10% FBS and incubated overnight in wells of multiscreen HTS 96-well filter plates (Millipore) that were previously coated with NP10–BSA. The following day, plates were washed of cells, and spots were detected with HRP-conjugated anti-mouse IgG (Southern Biotech) and developed with 3-amino-9-ethylcarbazole chromogen kit (Sigma) according to the manufacturer’s instructions.

B cell Ag presentation

Splenic cells (2 × 10⁵) from wild-type (WT) and Ncf1−/−/MD4 Ig-transgenic were pulsed with indicated concentrations of hen egg lysozyme (HEL)–OVA (gift from J. Cyster, University of California, San Francisco) for 2 h and washed to remove excess Ag. Ag-pulsed B cells were then incubated with 1 × 10⁶ CFSE-labeled OVA-specific OT-II TCR transgenic CD4+ T cells. After 72 h of coculture, cells were harvested and stained for CD19 and CD4, and OT-II proliferation was determined by monitoring CFSE dilution by flow cytometry, as described. Dead cells were excluded by propidium iodide uptake.

Results

BCR stimulation of primary mouse B cells induces the rapid production of ROS

Nonphagocytic cells are known to generate low levels of ROS in response to various stimuli such as growth factors or Ag receptor engagement (27). Using several assays that detect superoxide (O₂⁻) and/or hydrogen peroxide (H₂O₂), we observed that BCR stimulation of primary splenic B cells induced a measurable oxidative burst in response to BCR stimulation. For example anti-IgM F(ab')₂ stimulation induced the extracellular production of O₂⁻, which could be observed as early as 6 min and was maintained for at least 1 h after BCR stimulation, as measured by reduction of cytochrome c added to the outside of cells (Fig. 1A). In addition, stimulation of B cells with the diacylglycerol analogue PMA resulted in robust superoxide production, suggesting that BCR-induced ROS production by B cells is dependent on a DAG-stimulated signaling pathway downstream of the BCR (Fig. 1A). Similarly, stimulation of MD4 Ig-transgenic B cells (which express a transgenic BCR specific for HEL) with soluble HEL induced robust production of O₂⁻, whereas nontransgenic B cells stimulated with HEL showed no detectable response (Fig. 1B).

Highly reproducible, albeit low level, production of O₂⁻ in response to anti-IgM was also detected using a chemiluminescent oxidation-sensitive probe, in agreement with a previous report (21). The PMA-induced O₂⁻ burst detected by this assay was similar to what was observed by cytochrome c reduction (Fig. 1C).

Finally, as a measure of BCR-localized ROS production, we chemically conjugated anti-IgM F(ab')₂ Abs to the H₂O₂ detection probe, H₂DCFDA, and used this probe to stimulate splenic B cells as was recently reported (19). This method allows for flow cytometric measurement of local ROS production that is adjacent to the BCR. Superoxide generated extracellularly can be rapidly converted to H₂O₂, which is capable of diffusing across the plasma membrane into the cytoplasm (16) where it could act on cytoplasmic targets. In addition, this method can be used to demonstrate that ROS production is from B cells and not from contaminating phagocytic cells by costaining cells for surface markers to exclude non-B cell populations from the analysis. Using this reagent, we observed robust production of ROS specifically from mature splenic B cells as early as 5 min after BCR stimulation, whereas non-B cells did not show detectable fluo-

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** BCR stimulation of primary mouse B cells induces the rapid production of ROS. Purified mouse primary B cells (A–C) or total spleen cells (D), isolated from WT (A and C) or MD4/IgHEL (B) spleens, were stimulated for the indicated times with 0.5 μg/ml PMA, 25 μg/ml anti-IgM F(ab')₂, 1 μg/ml soluble HEL, or 20 μg/ml anti-IgM–DCFDA. (A) O₂⁻ production was measured by cytochrome c reduction from WT B cells that were unstimulated (open circles) or stimulated with PMA (filled squares) or with anti-IgM (filled squares). Relative O₂⁻ levels are shown as ΔOD 550–490 nm at 2-min intervals over 1 h. (B) O₂⁻ production by WT nontransgenic (filled squares) and WT/MD4 IgHEL (filled triangles) B cells stimulated with soluble HEL (solid symbols) or left unstimulated (open circles) was measured by cytochrome c reduction as in (A). (C) O₂⁻ production by WT B cells was detected using the chemiluminescent reagent Diogenes and is shown as relative light units (RLU) after stimulation with PMA (triangles) or anti-IgM (squares). (D) H₂O₂ production in the vicinity of the BCR was measured at the indicated time points by flow cytometry after activation with anti-IgM conjugated to DCFDA. H₂O₂ production was measured as fluorescence in the FITC channel at each time point and comparing levels in control B220⁻ (non-B cells) and B220⁺ CD93⁺ mature B cells. All data are representative of at least two to three independent experiments.
presence of this probe (Fig. 1D). Thus, ROS were produced in proximity to the BCR in a rapid and sustained fashion in response to BCR cross-linking.

Early BCR-induced ROS production is dependent on B cell expression of the Nox2-containing NADPH oxidase

Although phagocytic cells such as neutrophils are known to generate ROS utilizing the classical Nox2 isoform of NADPH oxidase, a number of additional isoforms of this enzyme have been identified and have been shown to have unique functions in a wide variety of tissues (13). Both the Nox2-containing NADPH oxidase as well as a calcium-regulated isoform, Duox1, have been suggested to be important for lymphocyte ROS production (12, 14, 20, 21). To investigate the source of BCR-induced ROS generation in primary murine B cells, we first assessed the expression of all known Nox/Duox catalytic subunit isoforms by semiquantitative RT-PCR. Expression of each isoform was compared with a positive control tissue to verify specificity of the PCR products. mRNA encoding the Nox2 “phagocyte oxidase” catalytic subunit was readily detected in B cells, whereas expression of other NADPH oxidase isoforms was very low or undetectable (Fig. 2A). The low-level amplification of Nox3 was also observed in control RNA samples that were not reverse transcribed, likely resulting from genomic DNA contamination (data not shown). These data are in agreement with results of transcript microarray data from the Immunological Genome Project (http://www.immgen.org).

We next asked whether the Nox2 phagocyte oxidase complex is responsible for BCR-mediated ROS production by using mice that harbor a loss-of-function mutation in Ncf1, which encodes the essential p47phox subunit of the Nox2-containing NADPH oxidase complex (23). Whereas p47phox is required for Nox2 activity, it is dispensable for other Nox/Duox isoforms (13). B cells from Ncf1-deficient mice showed no detectible O$_2$- production in response to PMA stimulation and a substantially reduced response to anti-IgM, as measured by cytochrome c reduction (Fig. 2B). In addition, B cells from Ncf1-deficient mice crossed to the MD4 IgHEL transgene failed to generate O$_2$- upon stimulation with soluble HEL, further indicating that activation of Nox2 downstream of BCR stimulation was mainly responsible for acute ROS production by B cells (Fig. 2B). These results are in agreement with a previous study (21) and our own independent observations (data not shown) showing that BCR-mediated ROS, detected by a chemiluminescent probe, were absent in mice genetically deficient in Nox2 (gp91phox). In addition, Ncf1-deficient B cells completely lacked early (5–20 min) production of BCR-localized ROS, as measured with anti-IgM–DCFDA, as well as intracellular production and/or accumu-

FIGURE 2. Early BCR-induced ROS production is dependent on B cell expression of the Nox2-containing NADPH oxidase. (A) Expression of NADPH oxidase catalytic subunit isoforms in purified splenic B cells was measured by semiquantitative RT-PCR using primers specific for Nox1–4 and Duox1–2. Shown are relative amounts of each transcript compared with a positive control tissue for each isoform. Wedges indicate 5-fold serial dilutions of cDNA (starting with 50 ng cDNA). (B) Top panel, O$_2^-$ was measured as in Fig. 1A by cytochrome c reduction from B cells isolated from spleens of WT (filled symbols) and Ncf1$^{-/-}$ (open symbols) mice stimulated with PMA (triangles) or anti-IgM (squares) for the indicated times. Bottom panel, O$_2^-$ was measured by cytochrome c reduction from B cells purified from WT/MD4 (filled triangles) or Ncf1$^{-/-}$/MD4 (filled squares) spleens and stimulated with soluble HEL for the indicated times. Basal ROS levels (open circles, top and bottom panels) were measured from unstimulated WT nontransgenic (top panel) or WT/MD4 (bottom) B cells. (C) H$_2$O$_2$ production within the vicinity of the BCR was measured by flow cytometry as in Fig. 1C from WT and Ncf1$^{-/-}$ B cells stimulated with 20 μg/ml anti-IgM–DCFDA for the indicated times. Shown are relative H$_2$O$_2$ levels from mature B cells (B220$^+$CD93$^-$), represented as a histogram for anti-IgM–DCFDA fluorescence. (D) Intracellular O$_2^-$ production by WT and Ncf1$^{-/-}$ B cells was measured by flow cytometry from cells loaded with the O$_2^-$ indicator dye MitoSOX Red and stimulated for the indicated times with 20 μg/ml anti-IgM. O$_2^-$ production by mature splenic B cells (B220$^+$CD93$^-$) was measured as fluorescence in the PE channel for the indicated time points.
lation of ROS after BCR stimulation as measured with the intracellular ROS detection probe MitoSOX Red (Fig. 2C, 2D). Although the latter reagent is generally used to detect mitochondrial ROS, in our hands this probe readily detected the early wave of ROS produced after BCR stimulation, which was highly dependent on the Nox2 NADPH oxidase (Fig. 2D).

Taken together, these results show that BCR stimulation induces rapid local production and intracellular accumulation of ROS that is highly dependent on B cell expression and activation of the Nox2 isoform of NADPH oxidase. Therefore, Ncf1-deficient B cells serve as an appropriate model for investigating the possible role for early B cell ROS production in regulating proximal BCR-dependent signaling events.

**Ncf1-deficient mice have relatively normal B cell development and maturation**

Because mutations that affect BCR signaling may influence B cell development and maturation, we looked at frequencies of mature and immature B cell subpopulations from Ncf1-deficient mice. Ncf1-deficient mice showed nearly normal ratios of pro-B and pre-B cells, newly formed B cells, and mature recirculating B cells in the bone marrow (Fig. 3B). Ncf1 deficiency did, however, result in a small but significant increase in the frequency of mature B cells and concomitant decrease in immature B cell populations in the spleen (Fig. 3C). We also looked at the frequencies of splenic B cell subpopulations in MD4-transgenic Ncf1-deficient mice, as subtle alterations in B cell development can sometimes be masked by changes in the repertoire of expressed BCRs. Fixing the BCR led to small changes in peripheral B cell frequencies, namely a small but significant increase in the percentage of immature T1 cells, as well as a decreased combined frequency of splenic marginal zone and B1 B cell populations (Fig. 3D). Although there were small changes in B cell subpopulation frequencies, deficiency in Nox2 NADPH oxidase activity had only modest effects on B cell development and maturation.

**Deficiency in Nox2 NADPH oxidase-dependent early ROS production does not alter proximal BCR signaling or downstream B cell activation and proliferation**

ROS molecules such as H$_2$O$_2$ have the ability to inhibit transiently phosphatase activity in the cytoplasm through oxidation of catalytic-site cysteine residues within these enzymes (11). Therefore, it has been proposed that production of ROS downstream of the BCR may function as a positive feedback signal by preventing phosphatases such as SHP-1 from inhibiting downstream BCR signaling (16). Because early BCR-induced ROS production was dependent on the Nox2 NADPH oxidase, we compared the magnitude of a variety of signaling reactions in B cells from WT and Ncf1-deficient mice. Ncf1-deficient B cells were similar to WT cells in their ability to flux calcium in response to BCR stimulation with anti-IgM (Fig. 4A). Furthermore, at several time points tested after anti-IgM stimulation (2–30 min), WT and Ncf1-deficient B cells had indistinguishable activation of proximal and downstream BCR signaling pathways, as measured by phosphorylation of the proximal tyrosine kinase Syk, as well as downstream signaling intermediates ERK and AKT (Fig. 4B and data not shown). Syk is thought to be a primary substrate for SHP-1-mediated dephosphorylation (5), so normal activation of this kinase in Ncf1-deficient B cells argues against a role for NADPH oxidase-derived ROS in inhibiting SHP-1 activity downstream of the BCR. Ncf1-deficient B cells were also indistinguishable from WT cells in activation-induced upregulation of the early and late activation markers CD69 and CD86 (B7-2) (Fig. 4C), and they also proliferated normally in vitro in response to BCR stimulation.
Deficiency in Nox2 NADPH oxidase-dependent ROS production does not alter proximal BCR signaling. Although our results, along with results of an independent study (21), indicate that early BCR-induced ROS production derived from the NOX1/2 NADPH oxidase complex is required for early BCR-induced ROS production independently of Nox2 NADPH oxidase activity. Our data show that early BCR-mediated production of Nox2-derived ROS does not enhance in vivo B cell Ag presentation and proliferation. (A) Cytoplasmic free calcium was measured by Indo-1 fluorescence emission ratio at 405 and 530 nm in WT (black lines) and Ncf1−/− (gray lines) follicular B cells (B220+, CD93−, CD23+, IgMint-lo) stimulated with 2 μg/ml (dashed) or 20 μg/ml (solid) anti-IgM. Data are representative of n = 2–3 mice each from two independent experiments. (B) BCR-induced phosphorylation of proximal (Syk) and downstream (ERK and AKT) targets was measured by flow cytometry in WT (solid black) and Ncf1−/− (dashed black) follicular B cells (B220+, CD24−, CD23+, IgMint-lo) stimulated with 25 μg/ml anti-IgM and stained intracellularly with p-specific Abs against activating phosphorylation sites of Syk, ERK1/2, and AKT. Basal phosphorylation status is represented by intracellularly stained unstimulated WT B cells (filled gray). Data are representative of n = 2–3 mice each from three independent experiments. (C) Upregulation of activation markers CD69 and CD86 was measured by flow cytometry from WT (solid black) and Ncf1−/− (black dashed) purified splenic B cells stimulated for 7 h (CD69) or 18 h (CD86) with 10 μg/ml anti-IgM. Data are representative of n = 2–3 mice in three independent experiments. (D) Proliferation of WT and Ncf1−/− B cells was measured by BrdU incorporation in vitro 48 h after stimulation with anti-IgM (10 μg/ml), CpG ODN (500 ng/ml), or anti-CD40 plus IL-4 (10 ng/ml). Proliferation was measured by intracellular staining with anti-BrdU and flow cytometry after an 18-h pulse with BrdU. The left panel shows representative histograms of BrdU+ WT (top) and Ncf1−/− (bottom) unstimulated and anti-IgM-stimulated B cells. Data are quantified in the right panel as the percentage of WT (white bars) and Ncf1−/− (black bars) that have incorporated BrdU. Data are representative of n = 3 mice/group from two independent experiments.

with anti-IgM, or BCR-independent stimulation with CpG, or CD40 and IL-4 (Fig. 4D). These results show that while the Nox2 NADPH oxidase complex is required for early BCR-induced ROS production, it is dispensable for BCR signal amplification and downstream activation and proliferation.

Deficiency in Nox2 NADPH oxidase-dependent ROS production does not alter T cell-dependent GC formation or B cell Ag presentation but enhances T cell-dependent Ab production

Although we did not observe any major differences in BCR signaling or activation of naive resting B cells in vitro, it is possible that subtle or activation-dependent differences in signaling may become more apparent in an in vivo setting such as during an Ab response. Surprisingly, Ncf1-deficient mice immunized with the T cell-dependent Ag NP-CGG produced somewhat elevated Ag-specific serum IgM and IgG1 titers (Fig. 5A). This difference did not appear to be due to differences in GC formation, as Ncf1-deficient mice had comparable numbers of IgDlo, Fas+, GL7+ GC phenotype B cells on days 7 and 14 after immunization (Fig. 5B). We also did not observe any differences in the numbers of NP-specific plasma cells measured by ELISPOT assay to detect anti-NP-IgG–producing Ab-secreting cells from spleens of day 14 immunized mice (Fig. 5C).

Previous studies have shown that Nox2 NADPH oxidase–dependent ROS generation is accompanied by ion and proton fluxes that promote Ag processing and presentation in dendritic cells (28–30). We were therefore curious whether Ncf1-deficient B cells had an altered ability to present Ag to cognate T cells. To test this possibility, Ncf1-deficient MD4 Iq-transgenic B cells were pulsed in vitro with various concentrations of a HEL–OVA conjugate and cocultured with OVA-specific OT-II TCR transgenic T cells to test their Ag presentation capability. Proliferation of OT-II T cells was measured by CFSE dilution as a readout of the cognate T cell response to Ag. On the basis of this assay, the Ag presentation ability of MD4 transgenic B cells was indistinguishable regardless of whether they had functional Nox2 activity or not (Fig. 5D). Thus, the reason that Ncf1-deficient mice generated somewhat higher titers of Ag-specific Ab was not evident.

Although we cannot rule out a B cell intrinsic effect on Ab production resulting from deficiency in Ncf1, these differences could be due to an absence of myeloid-derived ROS, which have been suggested to be immunosuppressive in some contexts (31, 32). In any case, these results indicate that early BCR-mediated production of Nox2-derived ROS does not enhance in vivo B cell responses to a T cell-dependent Ag.

BCR stimulation for 6 h or more leads to sustained production of ROS independently of Nox2 NADPH oxidase

Although our results, along with results of an independent study (21), indicate that early BCR-induced ROS production derived...
from the Ncf1<sup>−/−</sup> mice. Total numbers of GC B cells (IgD<sup>−</sup>, GL7<sup>+</sup>, FAS<sup>+</sup>) per spleen are shown from day 7 and day 14 NP-CGG immunized WT (white bars) and Ncf1<sup>−/−</sup> (black bars) mice. (C) Numbers of anti-NP–IgG–producing plasma cells per spleen from day 14 NP-CGG immunized WT (white bars) and Ncf1<sup>−/−</sup> (black bars) mice. (D) Ag presentation to cognate T cells by WT and Ncf1<sup>−/−</sup> B cells was measured by coculturing CFSE-labeled OT-II T cells with WT/MD4 (top panel) or Ncf1<sup>−/−</sup>/MD4 (bottom panel) B cells pulsed with increasing amounts of HEL-OVA. Presentation of OVA to cognate T cells was determined by measuring OT-II proliferation by CFSE dilution after 72 h of coculture. Data are representative of <i>n</i> = 3 mice/group from two independent experiments.

FIGURE 5. Deficiency in Ncf2 NADPH oxidase-dependent ROS production does not alter T cell-dependent GC formation or B cell Ag presentation but enhances T cell-dependent Ab production. (A) NP-specific IgM and IgG1 serum Ab titers were measured by ELISA from day 7 and day 14 NP-CGG immunized WT (open circles) and Ncf1<sup>−/−</sup> (filled circles) mice. Each circle represents an individual mouse (<i>n</i> = 6). Similar results were found in a second independent experiment. *<i>p</i> < 0.05, **<i>p</i> < 0.01. (B) GC formation in response to immunization with T cell-dependent Ag (50 μg NP-CGG in alum i.p.) in WT and Ncf1<sup>−/−</sup> mice. Total numbers of GC B cells (IgD<sup>−</sup>, GL7<sup>−</sup>, FAS<sup>+</sup>) per spleen are shown from day 7 and day 14 NP-CGG immunized WT (white bars) and Ncf1<sup>−/−</sup> (black bars) mice. (C) Numbers of anti-NP–IgG–producing plasma cells per spleen from day 14 NP-CGG immunized WT (white bars) and Ncf1<sup>−/−</sup> (black bars) mice. (D) Ag presentation to cognate T cells by WT and Ncf1<sup>−/−</sup> B cells was measured by coculturing CFSE-labeled OT-II T cells with WT/MD4 (top panel) or Ncf1<sup>−/−</sup>/MD4 (bottom panel) B cells pulsed with increasing amounts of HEL-OVA. Presentation of OVA to cognate T cells was determined by measuring OT-II proliferation by CFSE dilution after 72 h of coculture. Data are representative of <i>n</i> = 3 mice/group from two independent experiments.

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present in Ncf1-deficient B cells (Fig. 6A). By 24 h of stimulation, the levels of intracellular ROS seen in Ncf1-deficient B cells were nearly identical to those observed in BCR-stimulated WT B cells (Fig. 6B). This late production of ROS was measured in purified splenic B cells using the intracellular superoxide detection reagent MitoSOX Red, which has been reported preferentially to detect mitochondria-derived ROS (33). Pretreatment of cells with the ROS scavenger NAC blunted ROS production in WT B cells during the first 2 h and blocked further ROS production for at least 12 h. ROS production by Ncf1-deficient B cells was decreased compared with WT B cells, particularly at early times, and was completely blocked by 5 mM NAC (Fig. 6A, 6B). These data indicate that intracellular ROS detected by MitoSOX Red fluorescence was generated by Nox2 as well as by other mechanisms. The Nox2-independent production of ROS at later times was unlikely to be derived from other NADPH oxidase family members as BCR stimulation for 6 or 12 h did not induce expression of any additional Nox/Duox isoforms (Fig. 6C). In contrast, we found the production of ROS in BCR-stimulated B cells could be substantially enhanced by transient treatment with antimycin-A, which blocks complex III of the electron transport chain and leads to accumulation of mitochondria-derived O$_2^-$ (Fig. 6D) (34). This result indicates B cells stimulated via their BCR for 12 h have highly active mitochondrial respiration, which is known to be a significant source of intracellular ROS production in a variety of cell types (15). Taken together, these results demonstrate that B cell activation is characterized by a delayed production of ROS that is produced independently of the Nox2 NADPH oxidase and is maintained during prolonged BCR stimulation.

**Prolonged ROS production is required for optimal activation and proliferation and to sustain PI3K signaling in response to BCR stimulation**

As prolonged BCR signaling resulted in ROS production from a second source, we next asked if neutralizing the ROS generated by both mechanisms at later times would affect downstream B cell activation. Pretreatment of WT and Ncf1-deficient B cells with 5 mM NAC was sufficient to attenuate intracellular ROS accumulation substantially in WT B cells and to completely block it in Ncf1-deficient B cells (Fig. 6A, 6B). This dose of NAC resulted in significantly reduced BCR-induced activation as measured by upregulation of the early activation marker CD69 (Fig. 7A). Furthermore, this treatment resulted in a nearly complete block in proliferation of WT and Ncf1-deficient B cells stimulated with anti-IgM (Fig. 7B). Notably, NAC pretreatment did not impact proliferation in response to BCR-independent stimulation with anti-CD40 plus IL-4 or with CpG oligodeoxynucleotide (ODN) (Fig. 7B), indicating that the effect of 5 mM NAC on anti-IgM-induced proliferation was likely not due to toxicity, but rather that prolonged ROS production is a specific requirement for optimal responses to Ag receptor stimulation.
Treatment of WT and Ncf1-deficient primary B cells with 5 mM NAC had only minimal effects on proximal BCR signaling at early times, as determined by normal phosphorylations of Syk and ERK kinases in response to BCR cross-linking (Fig. 7D). A small reduction in BCR-induced calcium mobilization in both WT and Ncf1-deficient B cells was observed (Fig. 7C), but it was unclear whether this was due to scavenging of ROS or some other effect, and furthermore was unlikely to explain the near complete block in BCR-induced proliferation observed with 5 mM NAC. Higher concentrations of NAC (25 mM) further attenuated calcium signaling in agreement with a previous report (21) (data not shown); however, this likely reflects additional effects on B cells beyond simply scavenging ROS. As treatment of Ncf1-deficient B cells with 5 mM NAC clearly depleted all residual ROS produced over the first 6–12 h of anti-IgM stimulation (Fig. 6B) but did not substantially impact early BCR signaling, these results further support the conclusion that early production of ROS plays little if any role in amplifying proximal BCR signaling upon initial stimulation of resting B cells.

**FIGURE 7.** Prolonged ROS production is required for optimal B cell activation and proliferation and for sustained PI3K signaling in response to BCR stimulation. (A) Upregulation of the early activation marker CD69 was measured by flow cytometry 7 h after stimulation of WT (left) or Ncf1−/− (right) B cells with 10 μg/ml anti-IgM in the presence (black dashed) or absence (solid black) of 5 mM NAC. Data are representative of n = 3 mice/group. (B) Proliferation of WT and Ncf1−/− B cells in the presence or absence of NAC was measured by BrdU incorporation as in Fig. 3D. Data are represented as the total number of BrdU+ B cells 48 h after stimulation with anti-IgM (10 μg/ml), CpG ODN (500 ng/ml), or anti-CD40 (10 μg/ml) plus IL-4 (10 ng/ml) in the presence or absence of 5 mM NAC. **p < 0.01. Data from (A) and (B) are representative of B cells from n = 3 mice/group, and similar results were found in three independent experiments. (C) BCR-induced calcium mobilization was measured as in Fig. 4A in WT (top) and Ncf1−/− (bottom) follicular B cells stimulated with 25 μg/ml anti-IgM in the presence (dashed black lines) or absence (solid black lines) of 5 mM NAC. (D) BCR-induced phosphorylation of Syk and ERK was measured as in Fig. 4B in WT (left panels) and Ncf1−/− (right panels) follicular B cells stimulated with 25 μg/ml anti-IgM in the presence (dashed black) or absence (solid black) of 5 mM NAC. Data are representative of four (C) and three (D) independent experiments. (E) p-ERK and p-S6 levels were measured by intracellular staining and flow cytometry (as described in Fig. 4B) of purified WT B cells stimulated for 12 h with anti-IgM (10 μg/ml) in the presence (dashed black) or absence (solid black) of 5 mM NAC. (F) Quantification of p-S6 (represented as the percentage of p-S6+ cells) from purified WT B cells stimulated with anti-IgM, as in (E), in the presence or absence of NAC for the entire stimulation (12 h) or incubated for the last 2 h with NAC or 1 μM of the class I PI3K inhibitor GDC-0941. Data represent n = 3 mice/group and are pooled from two to four independent experiments. **p < 0.01 (Student t test).
To address the mechanism by which ROS promoted B cell activation and proliferation, we asked if this sustained ROS generation could be functioning to maintain signaling downstream of the BCR. After 12 h of anti-IgM stimulation, both WT and Ncf1-deficient B cells, pretreated with NAC to neutralize both mitochondrial and Nox2 NADPH oxidase-derived ROS, showed unaffected levels of ERK1/2 phosphorylation indicating intact MAPK signaling (Fig. 7E). However, signaling through the PI3K pathway was severely attenuated by neutralizing intracellular ROS, as phosphorylation of the ribosomal protein S6, a downstream readout of PI3K activity, was largely reduced by pretreatment with NAC (Fig. 7E). Phosphorylation of S6 at these late time points remained dependent on continuous PI3K signaling downstream of the BCR, as this signal was completely blocked by addition of the highly specific class I PI3K inhibitor GDC-0941 (35) for the last 2 h of stimulation (Fig. 7F). Importantly, p-S6 levels could also be similarly reduced by transient treatment with NAC for the final 2 h of stimulation (Fig. 7F). These results show that continuous signaling through the PI3K pathway requires the sustained production of ROS and that ROS-dependent amplification of this signaling pathway likely promotes efficient B cell activation and proliferation in response to BCR stimulation.

Discussion

In this study, we investigated the intracellular sources and biological function of ROS produced by primary B cells at early and late times after BCR stimulation. We found that the immediate production of ROS in resting B cells was dependent on the Nox2-containing NADPH oxidase complex, as B cells deficient in an essential component of this complex, p47\textsuperscript{phox} (Ncf1), were unable to generate detectable amounts ROS over the first several hours of BCR stimulation. However, examination of proximal BCR signaling, B cell activation, and B cell proliferation of BCR-stimulated Ncf1-deficient B cells indicated the lack of a unique role for early Nox2 NADPH oxidase-derived ROS production in these processes. Moreover, Ncf1-deficient mice mounted normal, or even elevated, Ab responses in vivo, further demonstrating that early BCR-induced ROS production does not function to enhance the initial response of B cells to Ag stimulation. In contrast, after several hours of BCR stimulation, a second major source of ROS emerged, likely generated as a product of mitochondrial respiration. Blocking accumulation of ROS at these later times with the ROS scavenger NAC resulted in severely attenuated PI3K signaling and led to marked defects in downstream B cell activation and proliferation after BCR stimulation. Thus, ROS did not substantially enhance proximal BCR signaling early after stimulation of resting B cells, but prolonged production of ROS played a critical role in enhancing BCR-mediated activation, at least in part, by promoting sustained BCR-induced PI3K signaling.

Our results demonstrating that BCR-induced early ROS production did not contribute measurably to the magnitude of BCR signaling are in agreement with a recent report (21) showing that B cells deficient in the catalytic subunit of Nox2, gp91\textsuperscript{phox}, also failed to generate ROS in response to BCR stimulation and had no significant defects in proximal BCR signaling. Furthermore, although both Ncf1-deficient mice (this study) and gp91\textsuperscript{phox} deficient mice (21) exhibit some alterations in Ab responses, these differences were modest in magnitude, and furthermore were elevated compared with WT mice with respect to levels of Ag-specific Ab titers. The reason for the enhanced T cell-dependent Ab response observed in Ncf1-deficient mice is not evident from these studies; however, it is possible that lack of Nox2 NADPH oxidase-derived ROS production in other immune cells is responsible for these differences. In particular, ROS produced by myeloid cells such as macrophages is known to have immunosuppressive effects on T cells during inflammation (31), and furthermore, regulatory T cells deficient in Nox2 have been shown to have reduced suppressive capacity (36, 37), which may be relevant to Foxp3\textsuperscript{T} T follicular regulatory cells that have recently been demonstrated to regulate GC responses (38, 39).

In contrast to our results with resting primary B cells, a previous study by Singh et al. (22) found that BCR stimulation of a B lymphoma cell line led to ROS production via the calcium-regulated NADPH oxidase isoform Duox1, resulting in transient inactivation of the PTPase SHP-1 and subsequent BCR signal amplification. A recent study also found that TCR stimulation of human T cell blasts induced rapid Duox1-dependent ROS production that was critical for early TCR signal amplification (20). We were unable to detect expression of Duox1 mRNA in primary resting B cells, and moreover, we did not detect any ROS production at early times after BCR stimulation of Ncf1-deficient resting B cells, indicating that Duox1 is not a significant contributor to BCR-induced ROS during the early activation of resting mature B cells. It is possible that expression of Duox1 is a feature of activated, GC, or memory B cells, or was acquired by the B lymphoma cell line used (A20) as one of the changes that contributed to immortalized growth and survival. Although we did not observe upregulated expression of Duox1 in BCR-activated B cells in vitro, it is possible that this may differ in an in vivo setting or in the context of B cell activation by other stimuli such as TLR ligands or Th cell-derived signals.

Another recent study examining B cells deficient in the proton channel HVCN1 suggested a positive signaling role for BCR-induced ROS production (19). HVCN1-mediated proton fluxes have previously been shown to be required for maintaining NADPH oxidase activity in neutrophils undergoing oxidative burst (40, 41). B cells deficient in HVCN1 showed a significant, albeit partial, reduction in ROS produced downstream of BCR stimulation, presumably due to the function of HVCN1 in transporting protons out of the cytosol to balance ionic movements created in the process of ROS production by NADPH oxidase. Notably, deficiency in HVCN1 also resulted in largely attenuated proximal BCR signaling and B cell proliferation in vitro and impaired Ab responses in vivo. These defects were attributed to a role for HVCN1 in B cells to maintain early NADPH oxidase-dependent ROS production that was required for transient inhibition of SHP-1 to promote BCR signal amplification. Our data showing that Ncf1-deficient B cells lack nearly all early BCR-induced ROS production but have no defect in BCR signaling or activation in vitro or in vivo indicate that the defects observed in HVCN1-deficient B cells probably reflect other mechanisms by which HVCN1 function contributes to proximal BCR signaling. As lack of HVCN1 in B cells resulted in cytosolic acidification and mitochondrial dysfunction, the BCR signaling and Ab response defects seen in HVCN1-deficient B cells could result from effects on a number of biochemical processes. Therefore, although an important role for proton translocation is identified by analysis of HVCN1-deficient B cells, the exact mechanism by which this contributes to BCR signaling remains to be defined.

Although Richards and Clark (21) and we found that the early Nox2 NADPH oxidase-dependent ROS production does not play a significant role in amplifying proximal BCR signaling, it is known that B cells require continual BCR stimulation for at least 24 h to enter the cell cycle and commit to cell division (42, 43). We found that BCR stimulation of naive resting B cells resulted in prolonged ROS generation, and by 6 h after anti-IgM stimulation a second source of ROS became significant that was independent of the Nox2 NADPH oxidase. Importantly, blocking accumulation...
of ROS from both sources during this time period with the antioxidative NAC severely impaired anti-IgM-induced activation and proliferation of both WT and Ncf1-deficient B cells. In contrast, this antioxidant did not inhibit proliferation induced by the TLR9 ligand CpG ODN or by anti-CD40 plus IL-4, demonstrating that the importance of ROS was restricted to activation through the BCR.

A potential source of Nox2-independent ROS in BCR-stimulated B cells is the mitochondria, which give rise to ROS in the form of \( \text{O}_2^- \) as an intermediate during the electron transport chain (15). Oxygen radicals are normally neutralized with high efficiency by enzymes in the mitochondria; however, a small percentage of this ROS is known to leak out of the mitochondria in the form of \( \text{H}_2\text{O}_2 \), which can diffuse through the mitochondrial membrane potentially to oxidize protein cysteine residues and thereby regulate signaling or other physiological processes in the cytosol. It is likely the ROS observed at these later times was derived from the mitochondria, as B cells were found to express negligible amounts of other NADPH oxidase isoforms both in the resting state or after BCR-induced activation in vitro. Moreover, ROS levels could be further enhanced by treating BCR-stimulated cells with a mitochondrial complex III inhibitor, a treatment that results in a buildup of \( \text{O}_2^- \) generated from the electron transport chain (44), indicating that activated B cells at these times had highly active mitochondrial respiration. Mitochondrial ROS have recently emerged as critical mediators of signaling in a number of contexts including macrophage cytokine production, inflammasome activation, and growth and proliferation of cancer cells (33, 45, 46). Our data indicate that the functions of mitochondrial ROS can likely be extended to signaling pathways important for B cell activation and proliferation.

The mechanism by which prolonged production of ROS enhanced B cell activation and proliferation is not totally evident from our studies; however, one likely contribution is enhanced activation of the PI3K signaling pathway, which is an important regulator of cell energy uptake and utilization (47, 48). This pathway is negatively regulated by the lipid phosphatase PTEN, which is known to be inactivated by ROS through oxidation of an active-site cysteine (8). We found that after 12 h of BCR stimulation, neutralizing ROS with the antioxidative NAC had little effect on sustained signaling through the ERK pathway, but this resulted in a marked reduction in phosphorylation of the ribosomal protein S6, which is consistent with an attenuation of upstream PI3K signaling. Furthermore, neutralization of ROS for just the last 2 h of stimulation in B cells treated for 12 h with anti-IgM also reduced S6 phosphorylation, indicating that the continued presence of ROS was required for maintaining this signaling pathway at these later times. We focused on S6 phosphorylation as a readout of PI3K activity in B cells due to the increased sensitivity of this signaling event compared with phosphorylation of AKT at these later times after BCR stimulation; however, it will be important to determine if other events downstream of PI3K activation are also similarly reduced by ROS neutralization. In particular, activated AKT is known to phosphorylate and inactivate the transcription factor FOXO1, which normally drives the transcription of genes that maintain cells in a quiescent state (49, 50), and this is consistent with our observation that neutralization of ROS with NAC prevented BCR-induced entry into the cell cycle.

Signaling through the PI3K pathway is critical for a number of cellular processes in lymphocytes including cell proliferation, survival, protein synthesis, and energy utilization, and therefore maintaining signaling through this pathway is likely essential for B cells to respond efficiently to cognate Ag (7, 47, 48, 51). Tonic low-level activation of this pathway is known to be required for the maturation and survival of naive B cells (52, 53); however, it has also been demonstrated that long-term maintenance of PI3K signaling is critical for B cell proliferation and survival after BCR stimulation (54). The production of ROS at these later stages of B cell activation may therefore represent a mechanism to promote signal amplification when BCR signaling is suboptimal due to moderate Ag affinity, reduced Ag concentration, and/or a reduced number of BCRs present on the cell surface after receptor internalization in response to initial Ag encounter. These results suggest that the amplification of late BCR signaling by ROS is likely critical for efficient B cell responses to T cell-independent Ags and/or for maintaining the activation status of Ag-stimulated B cells during the early stages of a T cell-dependent Ab response prior to receiving cognate-T cell help.

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