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# Kinetics of B Cell Receptor Signaling in Human B Cell Subsets Mapped by Phosphospecific Flow Cytometry<sup>1</sup>

Jonathan M. Irish,<sup>†‡</sup> Debra K. Czerwinski,<sup>†</sup> Garry P. Nolan,<sup>3‡</sup> and Ronald Levy<sup>2,3†</sup>

Differences in BCR signaling may govern outcomes as diverse as proliferation and cell death. We profiled BCR signaling kinetics in subsets of primary human B cells using flow cytometry. In the predominant population expressing IgM, BCR cross-linking led to a quick burst of Syk, ERK1/2, and p38 signaling. In contrast, IgG B cells sustained higher per-cell ERK1/2 phosphorylation over time. This dichotomy suggested a mechanism for dampening signals transmitted by IgM. Regulatory phosphatase activity in IgM B cells was BCR-mediated and initiated more slowly than kinase activity. This BCR-mediated phosphatase activity was sensitive to inhibition by H<sub>2</sub>O<sub>2</sub> and required to attenuate IgM BCR signaling. These results provide the first kinetic maps of BCR signaling in primary human B cell subsets and enable new studies of signaling in B cell disorders, such as autoimmunity and cancer. *The Journal of Immunology*, 2006, 177: 1581–1589.

B cell receptor signaling is intimately connected with B cell survival throughout development and must be finely tuned to produce effective humoral immunity. Expression of recombined IgH chain is required to provide survival signals from the pre-BCR (1, 2) and the BCR (3). Immature B cells can be selected by both positive and negative signaling for a lack of BCR affinity to self Ag (4) (e.g., clonal deletion (5) and receptor editing (6)), and maintained expression of the Ig $\alpha$  and Ig $\beta$  signaling subunits of the BCR is required for survival of mature B cells (7, 8). In mature B cells, signaling following Ag interaction at the BCR drives proliferation and expansion, and increased affinity of the BCR for Ag following somatic mutation appears to enhance these signals (9). B cells express M isotype IgH chain (IgM) throughout these processes that fine tune BCR signaling. Cytokine signaling and activation-induced cytidine deaminase activity enable B cells that have encountered Ag to undergo class switch from IgM to IgG (10, 11). Enhanced BCR signaling by an IgG-containing BCR may be one mechanism supporting the specialized role of memory B cells (12, 13). Changes in BCR signaling following class switch are thought to result from differences in binding of signaling regulators by IgM and IgG, and evidence indicates that recruitment of the cell surface coreceptor CD22 may be a key difference between IgM and IgG B cells (14, 15). Although BCR signaling must be constantly revised throughout B cell development, it has previously been difficult to study the impact these changes have on BCR-mediated signaling cascades in primary B cells.

Biochemical events initiated at the BCR have been mapped in detail over the last 10 years. BCR cross-linking by Ag triggers phosphorylation of tyrosines within the ITAM motif domains of Ig $\alpha$  and Ig $\beta$  by Src family member tyrosine kinases (e.g., Lyn, Lck, Blk, Fyn). The phosphorylated ITAMs of Ig $\alpha\beta$  recruit and enhance phosphorylation of Syk (directly) and Btk (via Syk). BCR cross-linking also brings together numerous regulator and adapter molecules (e.g., SLP-65/BLNK, Grb2, CD22, SHP-1) and compartmentalizes the BCR in lipid rafts with coreceptors CD19 and CD21. Following Syk and Btk activation, the enzymes phospholipase-C  $\gamma$  2 (PLC $\gamma$ 2)<sup>4</sup> and PI3K propagate BCR signaling. PLC $\gamma$ 2 activation generates calcium flux, inositol-1,4,5-triphosphate, and diacylglycerol, and results in activation of protein kinase C and NF- $\kappa$ B. Syk interacts with PLC $\gamma$ 2 via adapters, whereas Btk can interact directly, and each is required for PLC $\gamma$ 2 activity following BCR cross-linking (16, 17). Both Syk and Btk can activate PI3K following BCR cross-linking (18). Activation of PI3K enables Akt-mediated survival signaling, and PI3K is required for BCR-mediated survival during B cell development (19). PLC $\gamma$ 2 and PI3K also initiate kinase cascades that result in phosphorylation of the MAPK family proteins ERK1/2 and p38. Activation of the Ras-Raf-ERK1/2 signaling cascade is considered a central event in BCR signaling, and decreased Ras activation due to RasGRP1 and RasGRP3 loss in mouse impairs B cell proliferation (20, 21). In contrast, p38 is a stress response protein that interacts with p53 and regulates cell cycle checkpoints (21–23). Differential activation of ERK1/2 and p38 might enable the BCR to drive diverse cellular outcomes, but the question arises whether a given B cell activates these two pathways simultaneously or favors one pathway depending on additional signaling context.

Efficient activation of BCR signaling depends on generation of H<sub>2</sub>O<sub>2</sub> and inactivation of negative regulatory protein tyrosine phosphatases (PTPs). Following BCR cross-linking, recruitment and activation of calcium-dependent NADPH oxidases (NOX) proteins, such as NOX5, enables production of H<sub>2</sub>O<sub>2</sub> and lowers the signaling threshold for the BCR (24, 25). BCR-induced H<sub>2</sub>O<sub>2</sub> transiently inactivates membrane proximal PTPs, including SHP-1, via reversible oxidation of the catalytic cysteine to sulfenic acid. Elegant work reconstituting the BCR signaling pathway in

<sup>†</sup>Department of Medicine, Oncology Division, Stanford University, Stanford, CA 94305; and <sup>‡</sup>Department of Microbiology and Immunology, Baxter Laboratories for Genetic Pharmacology, Stanford University, Stanford, CA 94305

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<sup>2</sup> Address correspondence and reprint requests to Dr. Ronald Levy, Department of Medicine, Oncology Division, Stanford University, Stanford, CA 94305-5151. E-mail address: levy@stanford.edu

<sup>3</sup> R.L. and G.P.N. contributed equally to this work.

<sup>4</sup> Abbreviations used in this paper: PLC $\gamma$ 2, phospholipase-C  $\gamma$  2; PTP, protein tyrosine phosphatase; NOX, NADPH oxidase; MFI, median fluorescence intensity.

insect cells (26) has suggested a model of redox feedback loops where  $H_2O_2$  inactivates PTPs and enables amplification of early signaling events, such as Syk phosphorylation and ITAM binding (25). Recent work characterized endogenously generated  $H_2O_2$  as the primary redox species generated by BCR signaling and indicated that NOX-dependent production of  $H_2O_2$  was critical to initiate a wave of BCR signaling in mouse A20 B cells (24). Additional regulation of BCR signaling may be mediated by the BCR isotype. In mouse, the cytoplasmic tail of the IgG isotype BCR H chain does not recruit CD22, a coreceptor that can bind and negatively regulate IgM BCR H chain (15). However, CD22 can both positively and negatively regulate BCR signaling (14), so it is unclear how expression of different H chain isotypes might alter the signaling pathways activated by the BCR or the kinetics of BCR signaling.

Thus, significant prior work in immortalized B cell lines, murine transgenic models, and cell expression systems (26) has made the BCR signaling network one of the best understood signaling systems to date (27). However, BCR signaling has largely been defined *in vitro*. It is not clear how quickly the BCR activates effectors such as Syk and ERK1/2 in primary B cells, and it is unknown how long these molecules persist in an activated state. A key aim of this study was to measure BCR signaling events in individual primary human B cells. To monitor BCR signaling network activity we used phosphospecific flow cytometry (28, 29), which provides the capability to measure signaling biochemistry of endogenous proteins and measures multiple events in each cell. This technology also enables the observation of cell subsets with altered signaling in patient specimens and has numerous clinical applications (30–32). A better understanding of differences in BCR signaling kinetics in B cell subsets, such as IgM and IgG isotype B cells, is an important starting point for the study of signaling in B cell disorders. In addition to dissecting the kinetics of BCR-mediated signaling in primary cells, we examined differences in signaling activated by IgM and IgG BCRs and characterized a regulatory pathway in IgM B cells that is required to control BCR signaling output.

## Materials and Methods

### Isolation, storage, thawing, and equilibration of primary cells

PBMC were isolated using density gradient separation (Ficoll-Paque Plus; Amersham Biosciences). PBMC were pelleted by low-speed centrifugation, resuspended in medium composed of 90% FCS (HyClone) + 10% DMSO (Sigma-Aldrich), frozen slowly in the vapor phase of liquid nitrogen in multiple cryotubes, and stored in liquid nitrogen. For signaling analysis, an individual cryotube was thawed into 5 ml of Stem Span H3000 serum-free medium (StemCell Technologies), counted, pelleted, and resuspended at  $3.3 \times 10^6$  cells/ml. Thawed PBMC were allowed to rest at 37°C in a  $CO_2$  incubator for 2 h before stimulation.

### Stimulation of BCR signaling

At least half an hour before stimulation, 300  $\mu$ l of medium containing  $1 \times 10^6$  PBMC was aliquoted into flow cytometry tubes (Falcon 2052; BD Biosciences) and allowed to further rest at 37°C in a  $CO_2$  incubator. After resting, cells were stimulated by adding 6  $\mu$ l of a 0.5 mg/ml solution of the indicated F(ab')<sub>2</sub> to tubes, achieving a final concentration of 10  $\mu$ g/ml each F(ab')<sub>2</sub>. Cross-linking of B cell receptors was achieved using goat polyclonal anti-IgM and anti-IgG F(ab')<sub>2</sub> (BioSource International). When used,  $H_2O_2$  was at 3.3 mM final concentration (except where concentrations differed as indicated for titration) and was added as 2  $\mu$ l of a 500-mM stock solution immediately before BCR cross-linking. Time course studies of signaling kinetics were performed by adding stimulus to tubes individually in reverse time points and then fixing all samples in unison. During signaling, cells were kept in a 37°C  $CO_2$  incubator to allow signal transduction and phosphorylation. To determine basal levels of phosphorylation, unstimulated cells were maintained in parallel with stimulated cells and fixed at time zero. For fixation, 15  $\mu$ l of 32% paraformaldehyde (Electron Microscopy Services) was added to each 300- $\mu$ l tube of cells to a final concentration of 1.4%. Cells were fixed for 5 min at room temperature, pelleted, permeabilized by resuspension in 2 ml of methanol for 10 min, and stored at 4°C until being stained for flow cytometry.

### Intracellular phosphospecific flow cytometry

Paraformaldehyde-fixed, methanol-permeabilized cells were rehydrated by addition of 2 ml of PBS, gentle resuspension, and then centrifugation. The cell pellet was washed once with 2 ml of PBS + 1% BSA (Sigma-Aldrich) and resuspended in 50  $\mu$ l of PBS + 1% BSA. In some cases, samples were split evenly into two FACS tubes for parallel staining. A total of 50  $\mu$ l of an Ab mix containing primary conjugated phosphospecific Abs was added to each tube of cells, and staining proceeded for 20 min at room temperature. Phosphospecific alexa (Ax) dye Ax488 and Ax647 (Molecular Probes) or R-PE-conjugated Abs (all obtained from BD Pharmingen) were against phospho-Btk(Y551)/Itk(Y511), phospho-p38(T180/Y182), phospho-ERK1/2(T202/Y204), and phospho-Syk(Y352)/Zap70(Y319). Detection of cell subsets included FITC- and PE-conjugated polyclonal F(ab')<sub>2</sub> against IgM and IgG (BioSource International) and PercPCy5.5-conjugated Abs against CD20 cytoplasmic tail (clone H1; BD Pharmingen) and were applied in panels as shown (Table I). Notably, Abs for phosphorylated Syk also detect phosphorylated Zap70, and Abs for phosphorylated Btk also detect phosphorylated Itk. Zap70 and Itk are expressed in T cells and are thought to play a major role in T cell signaling as do Syk and Btk in B cell signaling. At least 5,000 events from each B cell subpopulation of interest were collected from each sample using a benchtop FACSCalibur dual-laser cytometer (BD Biosciences). For all flow cytometry plots, scale markings on axes indicate decades from  $10^0$  to  $10^4$  (labels have been omitted for space and clarity).

## Results

Four BCR signaling events were measured by flow cytometry as part of this study. Phosphorylated Syk and Btk were measured as upstream BCR-specific signaling events, whereas phosphorylated ERK1/2 and p38 were measured as functionally contrasting downstream signaling effectors. Phosphorylated isoforms of signaling proteins were detected by fluorophore-conjugated Abs. In a typical flow cytometry experiment, detection of CD20 expression (expressed on mature B cells) and BCR H chain isotype (here, IgM or

Table I. Ab panels for four-color flow cytometry measurement of signaling in B cell subsets

Comparison Being Made	Channel 1 (530 nm/30 BP)	Channel 2 (585 nm/42 BP)	Channel 3 (670 nm LP)	Channel 4 (661 nm/16 BP)
IgM vs IgG BCR isotype signaling	IgM	IgG	CD20	p-ERK1/2
B vs T cell signaling	p-p38	CD3	CD20	p-ERK1/2
Differential BCR signaling pathway activation in each B cell	p-p38	p-ERK1/2	CD20	p-Syk
IgM BCR kinetics, panel 1 <sup>a</sup>	IgM	p-ERK1/2	CD20	p-Syk
IgM BCR kinetics, panel 2 <sup>b</sup>	IgM	p-p38	CD20	p-Btk
Example fluorophore dye	Ax488 or FITC	PE	PercP-Cy5.5	Ax647

<sup>a</sup> Bp, band-pass wavelength; LP, long-pass threshold; P, phospho.

<sup>b</sup> Panel 1 and panel 2 indicate two different Ab-staining panels that together address IgM BCR kinetics by measuring different combinations of phosphoprotein.

IgG) was combined with detection of multiple phosphorylated signaling proteins to create flow cytometry staining panels (Table I).

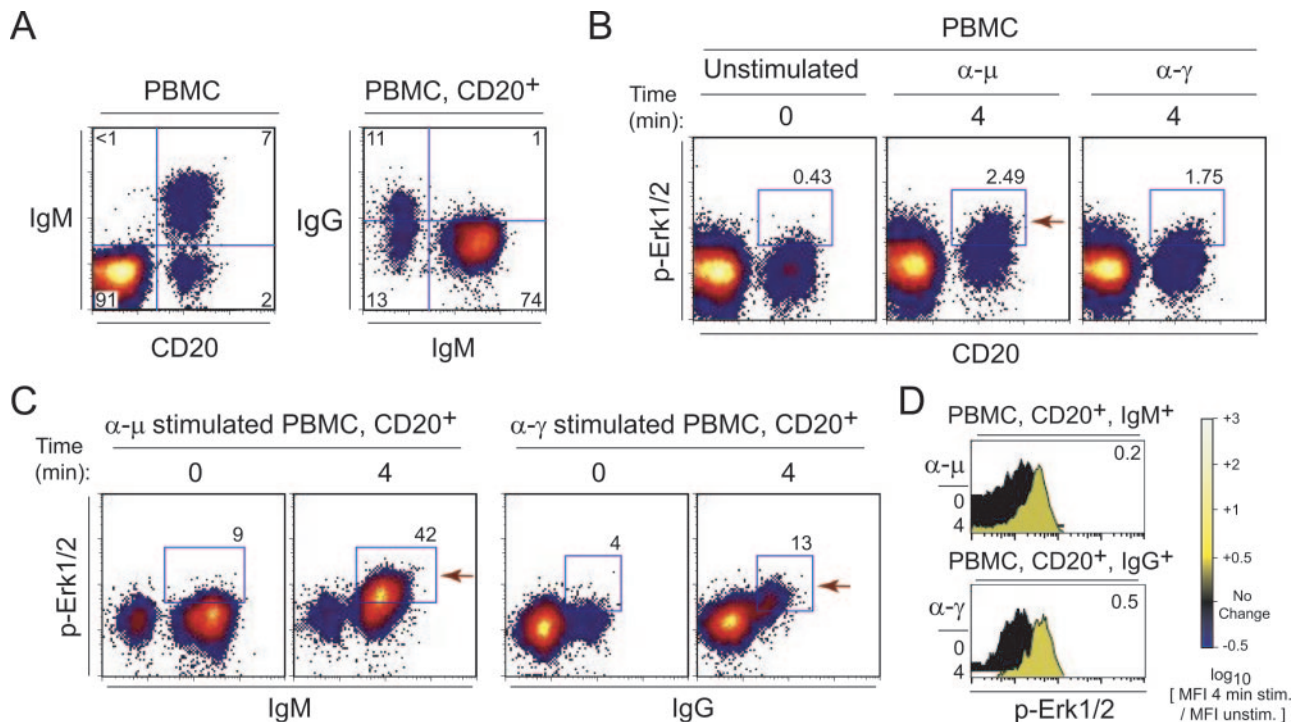
#### The BCR initiates distinct Syk and ERK1/2 signaling kinetics in IgM and IgG B cell subsets

PBMC were obtained from healthy blood donors, Ficoll purified, and cryopreserved. Cryopreserved PBMC samples were used so that results obtained here would be comparable to subsequent studies of cryopreserved samples from patients with B cell disorders (J. M. Irish, D. K. Gzerwinski, G. P. Nolan, and R. Levy, manuscript in preparation). Following thawing and equilibration, all the cells in a PBMC sample were exposed to stimulus together and the signaling resolved in cell subsets (Fig. 1). B cells were stimulated by cross-linking the H chain of the BCR using polyclonal F(ab')<sub>2</sub> specific for IgM ( $\alpha$ - $\mu$ ) or IgG ( $\alpha$ - $\gamma$ ). Stimulated PBMC were fixed and permeabilized, allowing detection of cell surface and intracellular epitopes. All Ab staining was performed subsequent to formaldehyde fixation and methanol permeabilization, which allowed detection of intracellular epitopes.

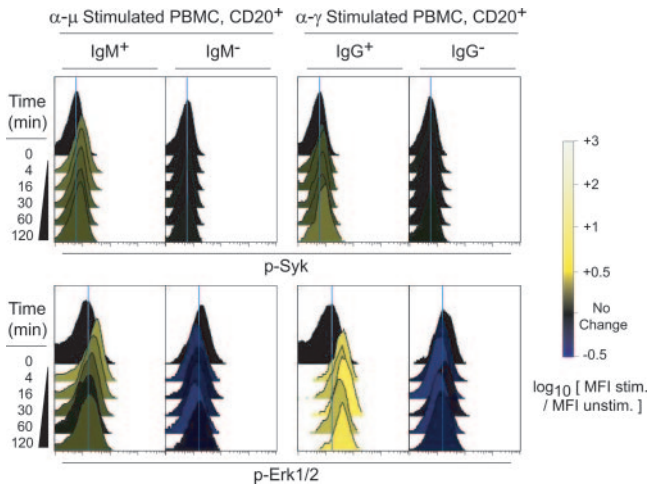
CD20<sup>+</sup> B cells typically constituted 5–10% of PBMC and were primarily IgM isotype (Fig. 1A). A small percentage of PBMC were IgG isotype CD20<sup>+</sup> B cells (<1%; Fig. 1A). At 4 min following  $\alpha$ - $\mu$  stimulation, modest ERK1/2 phosphorylation was detectable in the CD20<sup>+</sup> B cell subset of PBMC (Fig. 1B, arrow). When PBMC were gated as CD20<sup>+</sup> and the phosphorylation of ERK1/2 compared in IgM<sup>+</sup> and IgM<sup>-</sup> B cells (Fig. 1C),  $\alpha$ - $\mu$  stimulation was seen to be specific to IgM isotype B cells. Stimulation

by cross-linking IgG was difficult to discern within the total population without gating (Fig. 1B), because so few cells were IgG<sup>+</sup>. By gating the CD20<sup>+</sup> B cell subset and measuring ERK1/2 phosphorylation in IgG<sup>+</sup> and IgG<sup>-</sup> B cells, specific stimulation by  $\alpha$ - $\gamma$  was effectively resolved in this B cell subset (Fig. 1C). This induction of ERK1/2 phosphorylation in IgM and IgG cell subsets was compared using overlaid histograms colored according to the log<sub>10</sub>-fold change in median fluorescence intensity (MFI), relative to the unstimulated control (Fig. 1D).

Phosphorylation of ERK1/2 and Syk was measured at several times following BCR cross-linking of B cells of IgM or IgG BCR isotype to compare signaling kinetics (Fig. 2). Rapid, transient phosphorylation of Syk and ERK1/2 was detectable in IgM isotype B cells in the first 4–16 min following BCR cross-linking. In contrast, ERK1/2 phosphorylation in IgG isotype B cells immediately accumulated to a per-cell level that was higher than the maximum observed in IgM B cells and was sustained over 2 h (Fig. 2). As an internal negative control, cells negative for the BCR isotype being cross-linked were gated and are shown alongside the stimulated cells (e.g., IgM-negative cells in the  $\alpha$ - $\mu$  stimulated PBMC sample). In each case, no increase in Syk or ERK1/2 phosphorylation in the negative control cells was observed following BCR cross-linking of the opposite isotype. The transient burst of BCR-mediated signaling in IgM B cells contrasted with the sustained BCR signaling in IgG B cells and suggested that phosphatase activity may be required to dampen IgM BCR signaling.



**FIGURE 1.** Subset-specific stimulation of primary human B cells. *A*, Intracellular flow cytometry was used to detect B cell subsets in samples of human PBMC. The IgH chain isotype of CD20<sup>+</sup> cells was most commonly IgM. As a subset, IgG-expressing CD20<sup>+</sup> cells were <1% of PBMC. Numbers indicate the percentage of total cells in a region. *B*, Stimulation of ERK1/2 phosphorylation in specific B cell subsets within total PBMC was induced by cross-linking different BCR isotypes ( $\alpha$ - $\mu$  or  $\alpha$ - $\gamma$  F(ab')<sub>2</sub>, 4 min). Basal ERK1/2 phosphorylation in unstimulated cells was used as a control (0 min). Lack of induced ERK1/2 phosphorylation in other PBMC within the same tube was also used as a control (CD20<sup>-</sup> cells, 4 min). Arrows indicate significant ERK1/2 phosphorylation in B cell subsets. *C*, Phosphorylation of ERK1/2 in B cells of different H chain isotypes was compared following BCR isotype-specific stimulation ( $\alpha$ - $\mu$  or  $\alpha$ - $\gamma$  F(ab')<sub>2</sub>, 4 min). PBMC were gated as positive for CD20 expression, and then ERK1/2 phosphorylation in IgM<sup>+</sup> or IgG<sup>+</sup> B cells was measured. Note that intracellular flow cytometry detects both surface and intracellular epitopes, so subset-specific stimulation does not block subsequent detection of that subset but does lower the intensity of staining of that isotype. *D*, Histograms of ERK1/2 phosphorylation are shown for comparison of the fold change in phospho-ERK1/2 (p-ERK1/2) of IgM- and IgG-expressing B cell populations. The log<sub>10</sub>-fold change in MFI is indicated numerically and represented using a color scale, where increasing yellow to white color indicates increased phosphorylation and black indicates no change from unstimulated.

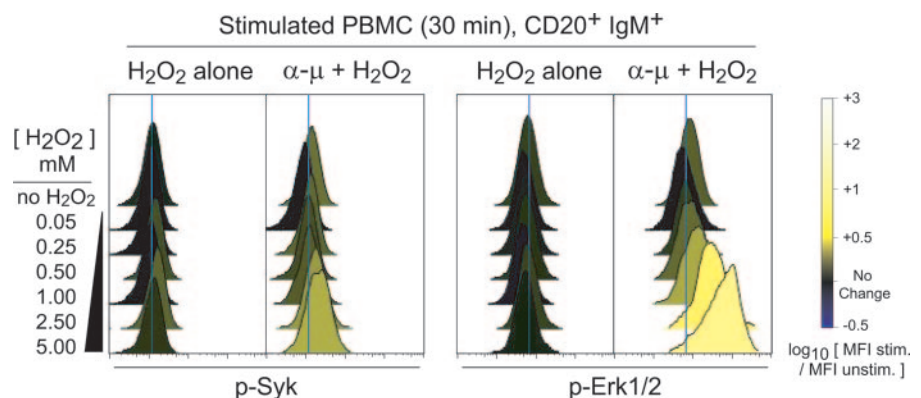


**FIGURE 2.** Contrasting IgM and IgG BCR signaling kinetics. PBMC were stimulated by  $\alpha$ - $\mu$  or  $\alpha$ - $\gamma$  BCR cross-linking for 4, 16, 30, 60, or 120 min and compared with unstimulated cells (0 min). CD20<sup>+</sup> PBMC B cells were then gated based on BCR isotype as either IgM or IgG, and the level of ERK1/2 and Syk phosphorylation was measured. Isotype-negative B cells provided an internal negative control. Histograms were colored according to the log<sub>10</sub>-fold increase in MFI of stimulated samples relative to unstimulated. A blue line has been drawn at the same value on each graph to compare the unstimulated MFI.

#### *H<sub>2</sub>O<sub>2</sub> amplifies and extends BCR signaling in primary B cells*

Phosphatase activity opposing BCR signaling in IgM B cells might be regulated by H<sub>2</sub>O<sub>2</sub>, the primary reactive oxygen species species produced by BCR signaling (24). CD20<sup>+</sup> IgM B cells were exposed to increasing concentrations of H<sub>2</sub>O<sub>2</sub> immediately before BCR cross-linking, allowed to signal for 30 min, and then compared with cells that were left unstimulated or stimulated by BCR cross-linking alone (Fig. 3). Addition of <5 mM H<sub>2</sub>O<sub>2</sub> without BCR cross-linking led to no significant phosphorylation detectable at 30 min in primary B cells. In contrast, greater than normal phosphorylation of Syk was observed in primary PBMC B cells when BCR cross-linking was combined with a concentration of 2.5 mM H<sub>2</sub>O<sub>2</sub> or greater. BCR-mediated ERK1/2 phosphorylation was sensitive to H<sub>2</sub>O<sub>2</sub> at concentrations of 1 mM or greater. H<sub>2</sub>O<sub>2</sub> combined with IgM BCR cross-linking led to much greater ERK1/2 phosphorylation at 30 min than that observed at 30 min following BCR cross-linking alone (Fig. 3). Based on the results of this titration, a concentration of 3.3 mM H<sub>2</sub>O<sub>2</sub> was used in subsequent experiments measuring kinetics of BCR-mediated signaling. These

**FIGURE 3.** Control of IgM BCR signaling is blocked by H<sub>2</sub>O<sub>2</sub>. Phosphorylation of Syk and ERK1/2 in CD20<sup>+</sup>IgM<sup>+</sup> PBMC B cells was measured at 30 min following various stimulations. The ability of IgM B cells to sustain BCR-mediated Syk and ERK1/2 phosphorylation induced by  $\alpha$ - $\mu$  cross-linking plus various concentrations of H<sub>2</sub>O<sub>2</sub> (right panels) was compared with the level of phosphorylation induced by H<sub>2</sub>O<sub>2</sub> alone (left panels). As additional controls, cells were left unstimulated (first peak, left panels) or stimulated by  $\alpha$ - $\mu$  only (first peak, right panels).



results indicated that phosphatase activity was important to regulating IgM BCR signaling, so phosphatase control of BCR signaling initiation, maximum activity, and duration were measured.

Phosphorylation of Btk, Syk, ERK1/2, and p38 was measured in primary CD20<sup>+</sup> B cells over a 2-h time course following BCR cross-linking, H<sub>2</sub>O<sub>2</sub>, or BCR cross-linking and H<sub>2</sub>O<sub>2</sub> (Fig. 4). B cells were stimulated by cross-linking BCRs of both IgM and IgG isotypes using a mixture of polyclonal F(ab')<sub>2</sub> raised against IgM and IgG. Histograms of Syk phosphoprotein expression in CD20<sup>+</sup> PBMC B cells are shown for one representative normal blood donor (Fig. 4A).  $\alpha$ - $\mu$ / $\gamma$  stimulation led to modest Syk phosphorylation that peaked between 4 and 8 min following BCR cross-linking and returned to basal levels by 90 min.

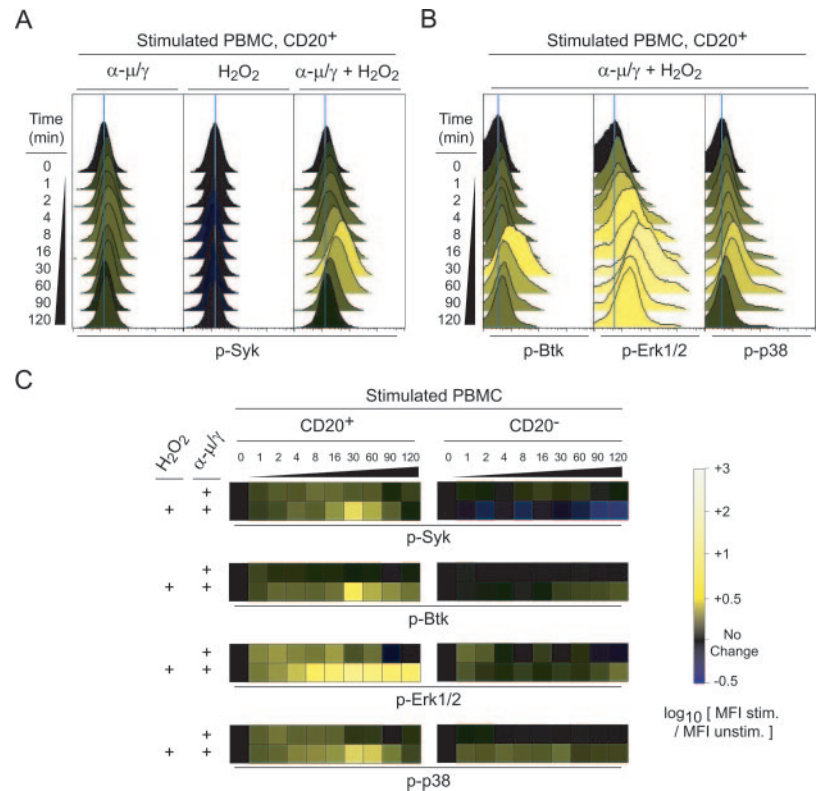
Treatment of PBMC with 3.3 mM H<sub>2</sub>O<sub>2</sub> led to no detectable Syk phosphorylation in CD20<sup>+</sup> B cells at any time over the 2 h following addition of H<sub>2</sub>O<sub>2</sub> to the medium (Fig. 4A). Addition of H<sub>2</sub>O<sub>2</sub> immediately before BCR cross-linking with  $\alpha$ - $\mu$ / $\gamma$  did not significantly alter the modest Syk phosphorylation observed during the first 4 min of signaling. However, at 8 min following H<sub>2</sub>O<sub>2</sub> +  $\alpha$ - $\mu$ / $\gamma$ , Syk phosphorylation continued to amplify and reached a peak at 30 min that was significantly greater than that observed following BCR cross-linking alone (Fig. 4A). BCR-mediated phosphorylation of signaling proteins Btk, ERK1/2, and p38 was also observed to be maximal between 30 and 60 min following stimulation by H<sub>2</sub>O<sub>2</sub> and BCR cross-linking (Fig. 4B). As a negative control, we examined the CD20<sup>-</sup> subset of PBMC, which were present in each tube of stimulated PBMC (Fig. 4C). CD20<sup>-</sup> cells in Ficoll-purified PBMC samples are typically a majority of T cells and minority of monocytes. Stimulation by  $\alpha$ - $\mu$ / $\gamma$ , H<sub>2</sub>O<sub>2</sub>, and  $\alpha$ - $\mu$ / $\gamma$  + H<sub>2</sub>O<sub>2</sub> did not cause significant increases in phosphorylation in CD20<sup>-</sup> non-B cells, as compared with CD20<sup>+</sup> PBMC B cells.

Comparison of phosphoprotein MFI following  $\alpha$ - $\mu$ / $\gamma$  or  $\alpha$ - $\mu$ / $\gamma$  + H<sub>2</sub>O<sub>2</sub> was used to assess the activity of H<sub>2</sub>O<sub>2</sub>. Addition of H<sub>2</sub>O<sub>2</sub> just before BCR cross-linking amplified and extended phosphorylation of Syk, Btk, ERK1/2, and p38 during the time when phosphorylation of each protein was normally observed to decrease following  $\alpha$ - $\mu$ / $\gamma$  alone (Fig. 4C). H<sub>2</sub>O<sub>2</sub>-sensitive phosphatases were therefore required to control the magnitude and duration of BCR signaling but not the early initiation of BCR signaling.

#### *Reproducible BCR signaling in B cells from different donors*

It is possible that very different signaling would be observed among different donors of normal blood. Differences in sample preparation and assay execution could also impact the observed BCR signaling kinetics. To assess variability in BCR signaling,

**FIGURE 4.**  $H_2O_2$  amplifies and extends BCR-mediated signaling. **A**, Phosphorylation of Syk in the  $CD20^+$  cell subset of PBMC was measured at 10 time points over 2 h following BCR cross-linking ( $\alpha\text{-}\mu/\gamma$ ),  $H_2O_2$  alone as a control, or combined stimulation ( $\alpha\text{-}\mu/\gamma + H_2O_2$ ). **B**, The kinetics of Btk, ERK1/2, and p38 phosphorylation were measured in the  $CD20^+$  B cell subset of PBMC following combined stimulation ( $\alpha\text{-}\mu/\gamma + H_2O_2$ ). **C**, Signaling over time was compared using a color scale based on the  $\log_{10}$ -fold increase in phosphorylation MFI in  $\alpha\text{-}\mu/\gamma$  or combination ( $\alpha\text{-}\mu/\gamma + H_2O_2$ ) stimulated cells, relative to the unstimulated control (0 min). As a control, the  $CD20^-$  cell population of PBMC, which is primarily  $CD3^+$  T cells and  $CD14^+$  monocytes, was gated separately, and the level of Btk, Syk, p38, and ERK1/2 phosphorylation was compared with that in the  $CD20^+$  B cell population.



multiple samples of peripheral blood from different individuals were prepared on different days, cryopreserved, and thawed for analysis of BCR signaling kinetics on different days. Phosphorylation of Btk, Syk, ERK1/2, and p38 were measured in  $CD20^+$  B cells from three different PBMC donors (D06, D07, D08) over a time period of 2 h following  $\alpha\text{-}\mu/\gamma + H_2O_2$ , and the absolute MFI of each phosphoprotein was determined by flow cytometry and plotted on a logarithmic scale of fluorescence (Fig. 5A).

The MFI of resting cells—a measure of the basal phosphorylation—was comparable from day to day and donor to donor (Fig. 5A). The average MFI and SD were calculated for each time point measured over 2 h following  $\alpha\text{-}\mu/\gamma + H_2O_2$  stimulation. BCR-mediated phosphorylation of Btk, Syk, ERK1/2, and p38 occurred at similar rates and reached similar per-cell levels in B cells from different blood donors (Fig. 5B).

#### *Initiation of BCR-mediated phosphatase activity is slower than kinase activity*

$H_2O_2$  is only transiently active before being scavenged by cellular thiols, and the inhibition of phosphatases by  $H_2O_2$  is reversible. We next asked whether the ability of  $H_2O_2$  to enhance BCR-mediated signaling was temporally limited.  $H_2O_2$  was added at various times to PBMC cells that were left unstimulated or stimulated by BCR cross-linking by  $\alpha\text{-}\mu/\gamma$  for 30 min (Fig. 6A). At experimental time zero,  $H_2O_2$  was added immediately before BCR cross-linking.

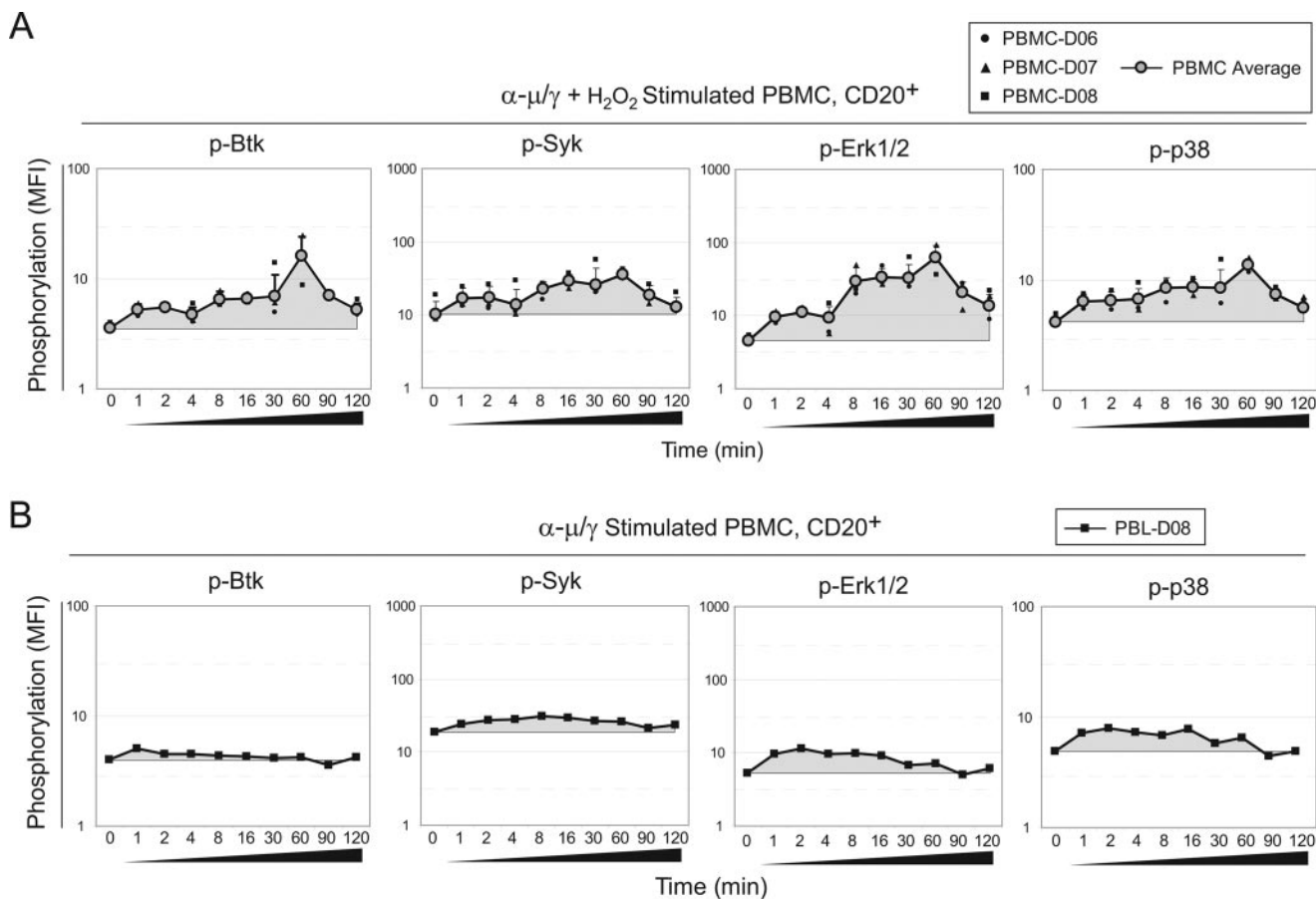
Addition of  $H_2O_2 > 1$  min before BCR cross-linking failed to enhance BCR-mediated Syk and ERK1/2 phosphorylation (Fig. 6). Addition of  $H_2O_2$  just before BCR cross-linking (0 min) amplified BCR-mediated Syk and ERK1/2 phosphorylation (Fig. 6C), consistent with prior experiments. However, addition of  $H_2O_2 > 1$  min following BCR cross-linking led to significantly greater Syk and ERK1/2 phosphorylation than addition immediately before BCR cross-linking (Fig. 6).  $H_2O_2$  only in-

creased Syk and ERK1/2 phosphorylation following BCR cross-linking and only affected signaling in B cells, as compared with the T cell population within the same sample (Fig. 6B). Syk and ERK1/2 phosphorylation were compared in individual  $CD20^+$  B cells from PBMC to assess whether phosphorylation events were occurring simultaneously in each cell.  $CD20^+$  B cells with greater Syk phosphorylation also displayed greater ERK1/2 phosphorylation (Fig. 6C). Thus, phosphatase activity controlling IgM BCR signaling was activated by BCR cross-linking and was initiated more slowly than BCR-mediated Syk and ERK1/2 phosphorylation.

## Discussion

These studies map the kinetics of four BCR signaling events in primary human B cells and characterize contrasting profiles of BCR signaling in IgM and IgG isotype B cells. A key advance of this study was the ability to stimulate and measure endogenous BCR signaling molecules in subsets of B cells within a heterogeneous pool of primary human cells. These results characterize BCR signaling in mature human B cells and underscore the importance of negative regulation in controlling BCR signaling in primary B cells.

We show in this study that the IgM BCR not only initiates immediate signaling via phosphorylation of Btk, Syk, ERK1/2 and p38, but also activates a delayed wave of negative regulatory phosphatase activity that is required to dampen phosphorylation of these proteins (Figs. 4–6). The results of these kinetic studies are shown in a summary model (Fig. 7). These results support the idea that BCR activation in primary human B cells initiates two competing waves of signaling: an initial BCR-mediated activation wave that results in immediate phosphorylation and activation of canonical BCR pathway members, such as Syk, Btk, ERK1/2, and p38; and a secondary wave of negative regulatory BCR-mediated signaling that is initiated more slowly and controls the magnitude and duration of the first wave. Inactivation of phosphatases by  $H_2O_2$ , the endogenous



**FIGURE 5.** B cell signaling kinetics are reproducible across multiple different blood donors. *A*, Signaling kinetics of Syk, Btk, ERK1/2, and p38 phosphorylation over 2 h were measured in the  $\text{CD}20^+$  B cell subset of PBMC from different normal donors (PBMC-D06, -D07, -D08, closed symbols). Samples were collected, stimulated, and stained on different days. The average MFI and the SD are also shown ( $\circ$  with error bars;  $n \geq 3$ ). The average MFI at time points 0, 4, 60, and 120 min includes additional PBMC samples from different donors (PBMC-D09, -D10;  $n = 5$ ). *B*, For comparison, phosphorylation of the same proteins following BCR cross-linking alone is shown on the same scale using the results from one PBMC sample (PBMC-D08).

redox species generated by BCR activation (24), indicated that negative regulatory phosphatase activity was normally required to control the kinetics of BCR signaling (Fig. 4).

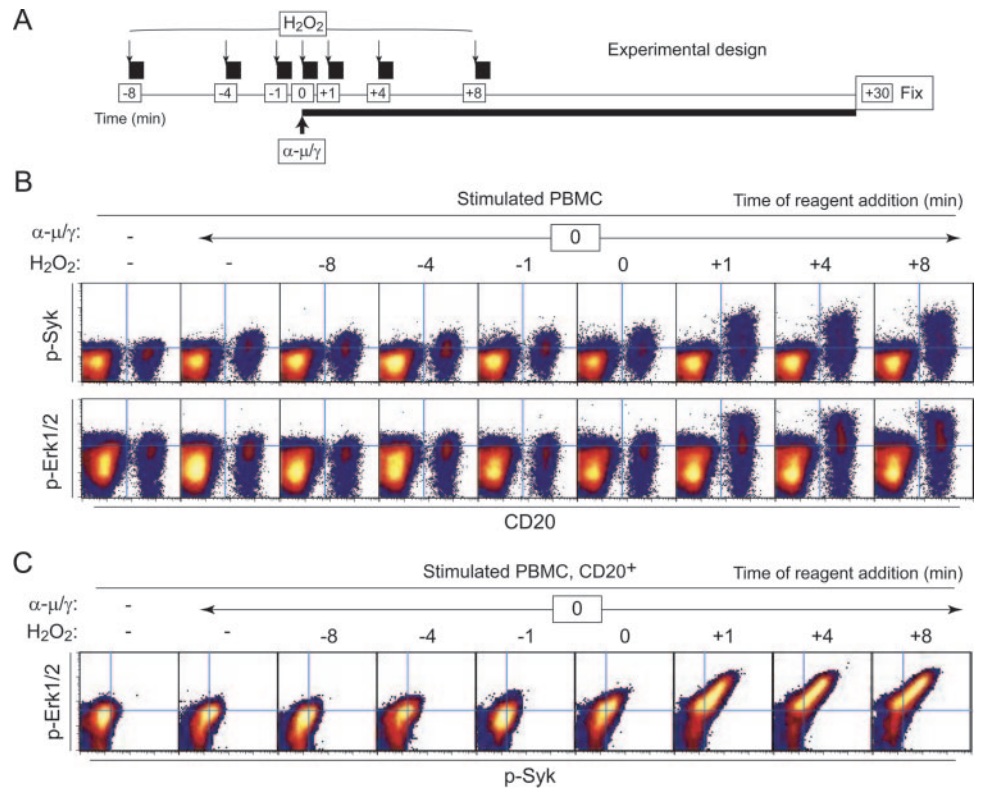
Measuring signaling kinetics allowed identification of two different ways the strength of BCR signaling is regulated in primary B cells. BCR-mediated signaling might be amplified by activating a greater number of the existing signaling molecules without changing activation and deactivation kinetics (i.e., more phosphorylated Syk per cell at a critical time). Alternatively, BCR signaling output might be altered by extending the time over which a given number of molecules in each cell were active, either by achieving maximal signaling more quickly or by extending the time at which maximum signaling is sustained. Our results show that B cells with an IgG BCR maintain ERK1/2 signaling over a significantly greater time than B cells with an IgM BCR (Fig. 2). In contrast, our results in IgM isotype B cells show that PTPs were activated to remove phosphorylation of Syk and ERK1/2 within 4–8 min following BCR engagement (Figs. 2 and 4).

This contrast between weaker, transient signaling kinetics in IgM isotype B cells and the robust signaling in IgG B cells supports the idea that class switch alters BCR signaling and indicates that key BCR signaling molecules, including Syk and ERK1/2, are regulated in an isotype-specific manner in primary human B cells. This regulation is comparable to that observed

in mouse B cells of IgG and IgM isotype (15). The sustained ERK1/2 phosphorylation over time that we observed in IgG isotype B cells identifies one way IgG B cells might achieve increased proliferation rates, relative to IgM B cells, following BCR engagement (12). Sustained BCR signaling in IgG isotype B cells might provide these cells with the capacity to have a robust and lasting signal for Ab production following exposure to Ag. Our results suggest that similar signaling output in IgM B cells can be obtained, but requires a signal to inactivate negative regulatory phosphatases, which are activated by the IgM BCR.

We show that phosphatase activity normally negatively regulates BCR signaling in human IgM B cells, and we show that this negative regulation is sensitive to levels of  $\text{H}_2\text{O}_2$  that do not initiate signaling in primary cells (Figs. 3, 4, and 6). Based on observations of mouse B cells (15), it seems likely that differences in affinity of IgM and IgG for CD22 may regulate phosphatase control of BCR signaling in human B cells. Our results also indicated that phosphatase activity does not control the initial accumulation of BCR signaling; phosphorylation of Syk, Btk, ERK1/2, and p38 increased at the same rate regardless of whether or not  $\text{H}_2\text{O}_2$  was present. Instead, phosphatases controlled the maximum levels of BCR signaling and the duration of BCR signaling. These results support the growing model of redox control of BCR signaling (24, 25). In this model, BCR-mediated signaling via  $\text{PLC}\gamma 2$  releases

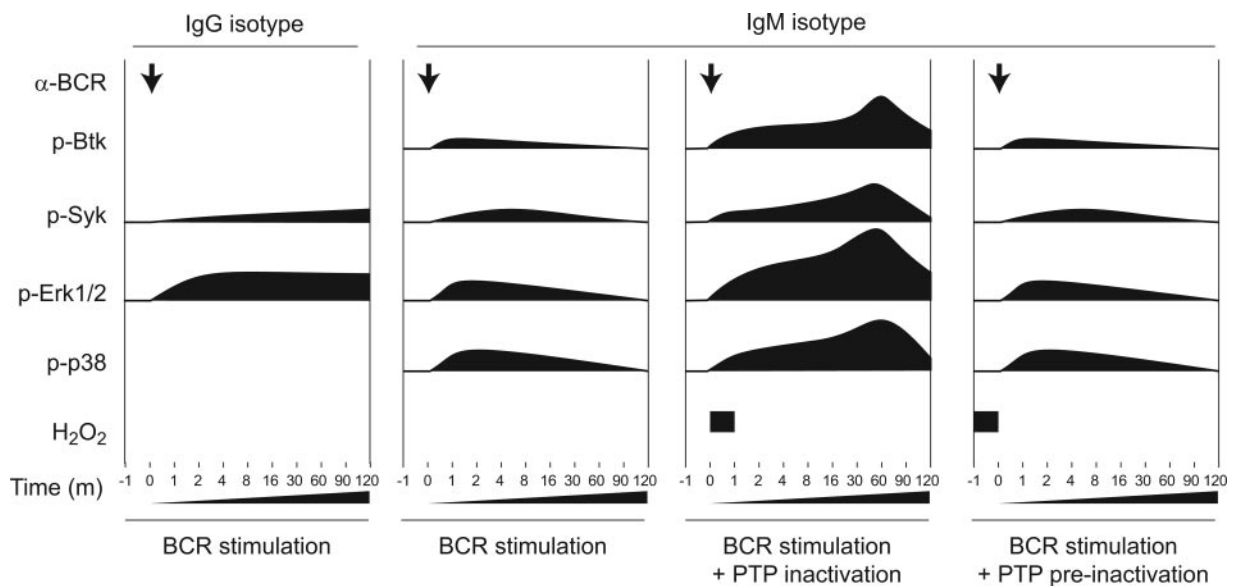
**FIGURE 6.** Delayed regulation of signaling is initiated by the BCR. *A*, Outline of the experimental design. PBMC cells were left unstimulated or exposed to H<sub>2</sub>O<sub>2</sub> at various times with and without BCR cross-linking by  $\alpha$ - $\mu/\gamma$ F(ab')<sub>2</sub>. All samples were fixed at 30 min following BCR cross-linking. H<sub>2</sub>O<sub>2</sub> inactivation of phosphatases is transient and reversible (represented by a short bar). *B*, The effect of adding H<sub>2</sub>O<sub>2</sub> at various times relative to BCR cross-linking ( $\alpha$ - $\mu/\gamma$ ) was determined by measuring phosphorylation of Syk and ERK1/2 in PBMC cells. Within a given PBMC sample, signaling in CD20<sup>+</sup> B cells was compared with that in CD20<sup>-</sup> cells. *C*, In the same experiment, PBMC were first gated as CD20<sup>+</sup> B cells. BCR-mediated phosphorylation of Syk and ERK1/2 was measured in each CD20<sup>+</sup> B cell to determine whether these events occur in the same cell.



intracellular calcium from the endoplasmic reticulum, activates a calcium-dependent NOX (e.g., NOX5), and generates H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> is thought to transiently and reversibly inactivate membrane-proximal PTPs and allow amplification of BCR signaling, but this has not previously been studied in primary human B cells. The kinetics of BCR signaling we observed in human IgM B cells support the

idea that H<sub>2</sub>O<sub>2</sub> can act as a second messenger by interrupting phosphatase activity and allowing the initial BCR-mediated activation signals to amplify (Fig. 7).

With these studies we were able to observe the timing and magnitude of BCR signaling in “real time” in primary B cells. Comparing these results to BCR signaling kinetics observed in



**FIGURE 7.** Control of BCR signaling magnitude and duration in primary human B cells. Following BCR ligation, an initial wave of activation takes place, leading to immediate detectable phosphorylation of upstream (Syk and Btk) and downstream proteins (ERK1/2 and p38) observed to peak within 2–4 min. A wave of PTP activity is also activated by BCR ligation in IgM-expressing B cells. The effects of this BCR-mediated PTP activity are detectable as early as 4 min, and by 60 min per-cell phosphorylation levels have returned to basal. H<sub>2</sub>O<sub>2</sub> can reversibly inactivate PTPs and here is effective over only a short time frame (~1 min). H<sub>2</sub>O<sub>2</sub>-mediated inactivation of PTPs inactivated BCR-mediated PTP activity at several times following BCR cross-linking, indicating that transient inactivation of negative regulatory phosphatases following BCR activation is enough to tip the balance of signaling outcomes between sustained activation and rapid shutdown.



B cell lines by immunoblotting (24) and by fluorescence resonance energy transfer (33), we see comparable, extremely rapid induction of Syk phosphorylation. A surprising result of these studies was that BCR-mediated accumulation of ERK1/2 and p38 phosphorylation occurred simultaneously with upstream activation of Syk and Btk phosphorylation (Figs. 2 and 4). It is possible that finer resolution of signaling kinetics over seconds instead of minutes might reveal distinct waves of upstream and downstream signaling. However, our results indicate that downstream signaling, especially in the case of IgG isotype B cells (Fig. 2), is not directly dependent on the level of Syk phosphorylation. Another surprising observation in this study was that BCR-mediated ERK1/2 phosphorylation was maintained much longer than phosphorylation of Syk, Btk, and p38 (Fig. 4). Syk and Btk proteins are required for BCR-mediated signaling during normal development (34–36). However, in these studies comparable levels of Syk and Btk phosphorylation preceded very different levels of ERK1/2 phosphorylation in IgM and IgG isotype B cells. Differential activation of downstream signaling pathways, such as Ras-Raf-ERK1/2-proliferative signaling vs p38 stress signaling, could depend on contributions from cell surface coreceptors such as CD19 and might be important in dictating the functional consequences of BCR signaling.

A key advantage of studying signaling in primary PBMC B cells is that the BCR signaling network proteins and their associated regulatory factors are expressed at endogenous levels. Following cross-linking of the BCR with F(ab')<sub>2</sub>, we observed that only modest, transient phosphorylation of BCR network proteins was observed in primary PBMC B cells (Fig. 4A). The low level of phosphorylation observed following cross-linking alone contrasted with higher per-cell phosphorylation of these proteins that we observed in B cell lines (J. M. Irish, unpublished data). In cell lines, H<sub>2</sub>O<sub>2</sub> alone has been observed to initiate phosphorylation of B and TCR signaling network proteins independent of ligand binding (Refs. 24, 26 and J. M. Irish, unpublished data). Thus, our results using human PBMC indicate two key ways in which BCR signaling is tightly regulated: per-cell phosphorylation level and degree of ligand-independent signaling.

These results also demonstrate that a subset of cells representing 1% of a mixed pool can be tracked by flow cytometry and their signaling distinguished from other cells (Fig. 1). Given the ability to detect Ag-specific B cell subsets, it would be straightforward to extend this analysis technique to study Ag-specific BCR signaling in primary B cells. This approach might be useful in mouse models, where endogenous Ag could be used as the stimulus and the signaling characterized at several stages of B cell development. Normal B cells are a diverse, polyclonal population, and the relationship between different BCR structure, signaling kinetics, and cell programming (via gene expression) is not well understood. Signaling kinetics in mixed pools of primary B cells could help to clarify changes to BCR signaling in human diseases, such as autoimmunity and cancer, as part of a signaling profile approach (32). To understand altered BCR-mediated signaling in an autoimmune or malignant B cell, it is also critical to understand the timing and magnitude of BCR-mediated signaling in different normal B cells. The consistent BCR signaling kinetics observed between B cells from healthy individuals (Fig. 5) allow for sensitive future comparisons of signaling between normal, autoimmune, and malignant B cells. The per-cell measurements made by flow cytometry will be especially valuable for such future studies of clinical tissues, because patient samples are typically composed of heterogeneous populations of primary cells that could each be distinguished for comparisons of signaling. Now, differences

in BCR-mediated signaling can be measured in individual B cells taken directly from a patient and compared (in each cell) with B cell isotype, idiotype, developmental stage, oncogene expression, and other biomarkers in a flow cytometry analysis of BCR signaling kinetics.

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## Disclosures

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