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### **Proliferation and Survival of Activated B Cells Requires Sustained Antigen Receptor Engagement and Phosphoinositide 3-Kinase Activation<sup>1</sup> ✓**

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# Proliferation and Survival of Activated B Cells Requires Sustained Antigen Receptor Engagement and Phosphoinositide 3-Kinase Activation<sup>1</sup>

Amber C. Donahue and David A. Fruman<sup>2</sup>

**In this study, we investigate the extracellular and intracellular signals that drive cell cycle progression of activated B cells in the absence of T cell help. We find that brief engagement of the B cell receptor is sufficient to induce a single cell division in a fraction of cells, but that survival during successive cell divisions requires sustained receptor stimulation. In contrast, T cells have been shown previously to commit to multiple cell divisions following brief TCR engagement. Both early and late B cell receptor signals are blocked by inhibitors of phosphoinositide 3-kinase and mammalian target of rapamycin and are associated with S6 kinase activation and increased cell size. The requirement for ongoing Ag receptor signaling can be overcome by engagement of CD40 but only partially by IL-4. Proliferation driven by LPS also requires sustained exposure to the stimulus. These findings reveal checkpoints that may limit T-independent B cell responses when Ag exposure is transient. *The Journal of Immunology*, 2003, 170: 5851–5860.**

**C**lassical T-dependent (TD)<sup>3</sup> Ags are monomeric, oligomeric, or haptenated proteins with limited ability to cross-link the B cell receptor (BCR). Processing and presentation of peptides from TD Ags to effector T cells stimulates the production of cytokines (e.g., IL-4) and surface expression of CD40 ligand, which act back on the B cell to drive cell cycle progression and differentiation. T-independent (TI) Ags are generally classified into two types. TI-1 Ags stimulate B cells not through the clonally variant BCR but through germline-encoded pattern recognition receptors such as Toll-like receptors (TLRs). TI-2 Ags are polyvalent structures, such as bacterial cell wall components, that have the ability to strongly cross-link the BCR. Responses to TI Ags, especially TI-2, are generally augmented further by T cell help. An *in vitro* correlate of a TD response is the stimulation of primary murine splenic B cells with anti-CD40 plus IL-4. LPS, which at high concentrations stimulates polyclonal activation of B cells through a complex containing TLR4 (1), is used as a model TI-1 Ag. Both of these conditions trigger vigorous B cell proliferation and differentiation. Stimulation with a cross-linking anti-IgM Ab simulates a TI-2 response and induces moderate polyclonal B cell proliferation that is accompanied by a high rate of apoptosis (2, 3).

Upon encountering Ag, resting B cells exit the G<sub>0</sub> phase of the cell cycle, grow in size, and enter S phase (DNA synthesis) 24–48 h later. It has been reported that commitment to S phase requires exposure to anti-IgM for at least 24 h (4, 5). In contrast, it was recently shown that engagement of the TCR for as little as 2 h is sufficient to trigger cell division in Ag-specific T cells (6–9).

These findings would suggest that regulation of proliferation differs in B and T cells activated via the Ag receptor. This may be due to the production by activated T cells of autocrine growth factors, such as IL-2, that drive progression through G<sub>1</sub> and into the S phase. Interestingly, use of the cell division tracker dye CFSE revealed that the brief TCR engagement was sufficient to drive not only the first mitotic event, but multiple successive cell divisions in T cells (6–9). Although CFSE has been used to correlate B cell differentiation with cell division history (10), the duration of BCR engagement required to drive successive cell divisions in B cells has not been investigated using this approach.

One of the early signals required for B cell proliferation is the activation of phosphoinositide 3-kinase (PI3K) (11). Anti-IgM and LPS have each been shown to induce activation of PI3K within minutes of B cell stimulation (12, 13). In the case of BCR signaling, PI3K plays a critical role in the generation of a sustained calcium flux (11). In addition, PI3K signaling leads to activation of several serine/threonine kinases, including Akt and S6 kinase (11). LPS does not induce calcium flux (14) but does trigger the PI3K-dependent activation of NF- $\kappa$ B (13). It is not known whether PI3K signaling is involved at later stages of cell cycle progression following exposure to anti-IgM or LPS. In T cells, IL-2 activates PI3K and this provides an essential survival signal and contributes to the cell cycle program (15). Indeed, PI3K inhibitors block T cell proliferation when added 9 h after formation of T cell/APC conjugates (16).

In this study, we tested the hypothesis that PI3K signaling is required not just for the initial activation of B cells, but also during cell cycle progression. The data indicate that PI3K activity is required at multiple points during the expansion of activated B cells. We also determined the duration of Ag receptor stimulation required for productive B cell activation. The results show that sustained or recurrent Ag receptor signaling provides a necessary survival signal to allow B cells to complete multiple divisions.

## Materials and Methods

### Mice

BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Charles River Breeding Laboratories (Wilmington, MA) and bred in our colony. Animal use was approved by the institutional animal care and use committee.

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<sup>3</sup> Abbreviations used in this paper: TD, T dependent; BCR, B cell receptor; TLR, Toll-like receptor; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; EtOH, ethanol; S6K1, p70S6kinase; TI, T independent.

## Cells

Single-cell suspensions were obtained from the spleens of 6- to 10-wk-old mice and depleted of RBCs by hypotonic lysis. Purified B cells were obtained, as described previously (3), by negative selection with magnetic beads specific for CD43 (Miltenyi Biotec, Auburn, CA). B cell purity was determined to be >96% by FACS analysis (FACSCalibur and CellQuest Software; BD Biosciences, Mountain View, CA) using anti-B220 and anti-Thy1.2 Abs (BD Biosciences). For all assays, cells were stimulated in RPMI 1640 supplemented with 10% heat-inactivated FCS, 5 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ M 2-ME.

## Proliferation assays

Purified B cells ( $5 \times 10^4$ /well) were stimulated in triplicate in 100  $\mu$ l of total volume in 96-well flat-bottom dishes. Stimuli were used at the following final concentrations: goat anti-mouse IgM (F(ab')<sub>2</sub>, 10  $\mu$ g/ml; Jackson ImmunoResearch Laboratories, West Grove, PA), LPS (serotype 0127: B8, 10  $\mu$ g/ml; Sigma-Aldrich, St. Louis, MO), hamster anti-mouse CD40 (1  $\mu$ g/ml; BD Biosciences), and recombinant murine IL-4 (2 ng/ml; Endogen, Woburn, MA). [<sup>3</sup>H]Thymidine was added at 48 h (1  $\mu$ Ci in 50  $\mu$ l of medium/well) and 16 h later plates were harvested (MachII-96; TomTec, Hamden, CT) onto filters and counted in a BetaPlate scintillation counter (Wallac, Gaithersburg, MD).

## Inhibitors

In all assays where pretreatment with pharmacological inhibitors is indicated, cells were incubated for 15 min at 37°C with a 2 $\times$  concentration of inhibitor. Following addition of stimuli to cells, the final concentration of inhibitor was 1 $\times$ . Blocking Abs were prepared at 20 $\times$ , and pretreatment consisted of adding 5  $\mu$ l of Ab to the well just before the addition of cells. For all posttreatment conditions, both types of inhibitor were added directly to the wells at the indicated times. Pharmacological inhibitors were used at the following final concentrations: LY294002 (5  $\mu$ M), wortmannin (25 nM), rapamycin (10 nM), and FK506 (10 nM) (Calbiochem, La Jolla, CA). Blocking Abs used were goat anti-mouse IgM (Fab, 10–50  $\mu$ g/ml; Jackson ImmunoResearch Laboratories) and monoclonal TLR4 Ab (0.1–1  $\mu$ g/ml; eBioscience, San Diego, CA).

## Washout conditions

In those assays where stimuli were washed out at a given time, at least double the number of cells needed for replating were stimulated in larger volumes under conditions identical to the thymidine assays. At the indicated times, cells were removed from culture, pelleted by centrifugation, washed twice with 2 ml of medium, and counted. They were then restimulated with fresh stimulus or medium alone at the given concentrations under identical cell number and volume conditions as the corresponding continuously stimulated samples.

## Cell cycle and size analysis

Purified B cells ( $1.5 \times 10^5$ ) were cultured in 300  $\mu$ l of total volume in 48-well dishes under the stimulation and inhibition conditions described for thymidine incorporation assays. At the indicated times, cells were removed from culture, resuspended in 250  $\mu$ l of 1 $\times$  PBS/5 mM EDTA, and fixed by adding an equal volume of cold 100% ethanol. After 30 min at room temperature, cells were pelleted by centrifugation and resuspended in 250  $\mu$ l of 1 $\times$  PBS/5 mM EDTA. RNase A (100  $\mu$ g/ml) was added and tubes were incubated for 30 min at room temperature. Two hundred fifty microliters of 1 $\times$  PBS/5 mM EDTA containing 0.1 mg/ml propidium iodide was added directly before analysis by flow cytometry (FACSCalibur). The percentages of cells in different phases of the cell cycle were calculated with ModFit LT software (Verity Software House, Topsham, ME). Mean cell size information was obtained from the forward scatter data acquired using CellQuest.

## Immunoblot

Purified B cells ( $5 \times 10^5$ ) were cultured in 24-well dishes. Cells were harvested at the indicated times, pelleted, washed twice with 1 $\times$  PBS, and snap frozen. Frozen pellets were thawed and then lysed in 1 $\times$  SDS-PAGE sample buffer and heated at 95°C for 5 min. Samples were resolved on 8% gels and transferred onto nitrocellulose membranes. Membranes were incubated at least 1 h in blocking solution containing 1 $\times$  TBST and 5% nonfat dry milk and then immunoblotted with a rabbit antiserum against S6K1 (gift from K. Lee-Fruman). Bound Abs were visualized by incubation with HRP-conjugated goat anti-rabbit IgG (Promega, Madison, WI), followed by ECL detection (Pierce, Rockford, IL).

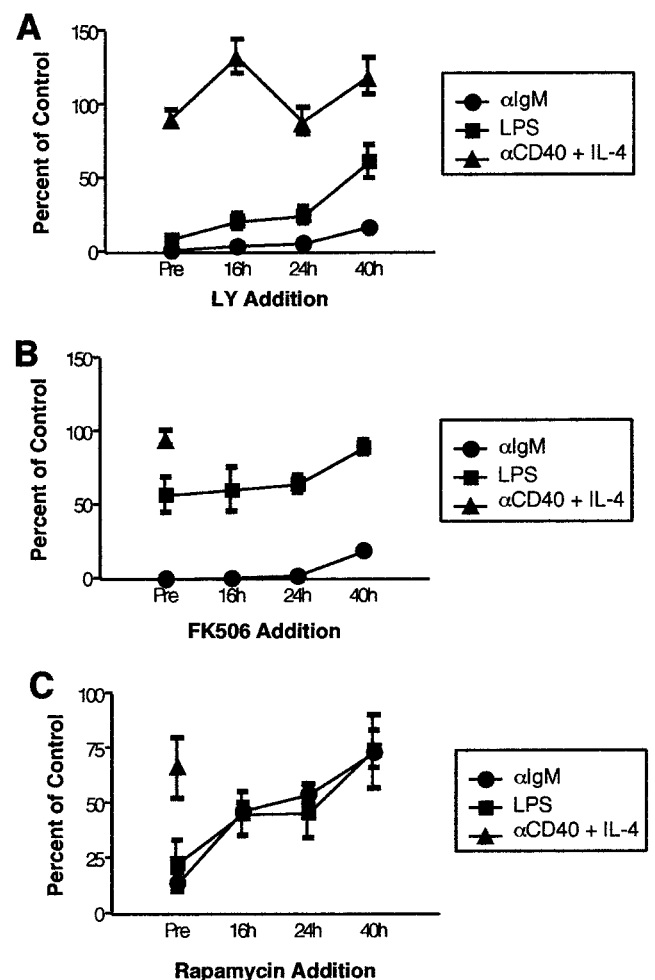
## CFSE

Purified splenic B cells were resuspended to  $10^7$ /ml in HBSS (Cellgro, Herndon, VA)/0.1% BSA (EM Science, Gibbstown, NY). CFSE (Molecular Probes, Eugene, OR) was added to a final concentration of 10  $\mu$ M and samples were vortexed and incubated for 10 min at 37°C. Cells were then washed once with a 10 $\times$  vol of HBSS/0.1% BSA and twice with a 10 $\times$  vol of medium. Cells were counted and stimulated as described. Data from 40,000 total events were acquired and analyzed using CellQuest.

## Results

### Requirement for PI3K, mammalian target of rapamycin (mTOR), and calcineurin in early and late signaling

PI3K activity increases within seconds of cross-linking the BCR and within minutes following addition of LPS (12, 13). This activation has been assumed to be necessary for entry into the cell cycle, as B cells lacking PI3K function show greatly reduced proliferation (3, 17). To determine whether PI3K function might also be required at later time points in activated cells, we added the specific PI3K inhibitor LY294002 at various times before and after stimulation of purified splenic B lymphocytes (Fig. 1A). Thymi-



**FIGURE 1.** Inhibition of PI3K, calcineurin, or mTOR at late time points reduces B cell proliferation. B cells activated with the indicated mitogens were either pretreated (Pre) or posttreated at the indicated times with LY294002 (A), FK506 (B), or rapamycin (C) or vehicle (EtOH). Data are expressed as the percentage of vehicle control. Shown is the mean  $\pm$  SEM of five experiments. Actual vehicle controls for anti-IgM (16,000, 25,000, 17,000, 59,000, 66,000 cpm), LPS (20,000, 87,000, 55,000, 133,000, 320,000 cpm), and anti-CD40 + IL-4 (95,000, 121,000, 145,000, 241,000, 341,000 cpm).

dine incorporation from 48 to 64 h was used as a measure of cell proliferation. Pretreatment with the inhibitor reduced proliferation induced by anti-IgM and LPS by 100 and 90%, respectively, consistent with previous work (3, 17, 18). Notably, addition of the drug at 16, 24, and as late as 40 h after stimulation also attenuated proliferation. In the case of anti-IgM, nearly 80% inhibition was still observed when the drug was added at 40 h compared with the diluent-treated control. For LPS, the drug was nearly as effective at 16 or 24 h as it was in pretreated cells, but caused only ~40% inhibition at 40 h. The patterns of inhibition by a distinct PI3K inhibitor, wortmannin, were similar although less complete (our unpublished observations), consistent with the short half-life of this compound in culture. Cells stimulated with a combination of anti-CD40 plus IL-4 and treated with LY294002 (Fig. 1A) or wortmannin showed similar levels of thymidine incorporation to those treated with diluent control, showing specificity and lack of toxicity of the drugs at the concentrations used. These data suggested a requirement for sustained or recurrent PI3K activation upon stimulation with anti-IgM or LPS.

Inhibitor studies have also suggested a late requirement for both mTOR and calcineurin activity in activated murine B cells (19). Similarly, we found that addition of the calcineurin inhibitor FK506 to anti-IgM-stimulated cells reduced proliferation, with ~80% inhibition relative to the diluent-treated control even at 40 h (Fig. 1B). The LPS response was also inhibited by pre- or post-treatment with FK506, but to a lesser degree (Fig. 1B). The addition of the mTOR inhibitor rapamycin led to a similar pattern of inhibition between anti-IgM- and LPS-stimulated B cells (Fig. 1C). Pretreatment was ~90% effective at blocking proliferation, with later additions decreasingly effective. Pretreatment of cells stimulated with anti-CD40 plus IL-4 demonstrated that FK506 and rapamycin were not toxic at the concentrations used. Similar effects of LY294002, FK506, and rapamycin were seen in B cells from a different mouse strain (C57BL/6) (our unpublished observations).

To determine the kinetics of cell cycle entry in stimulated B cells, and to determine the importance of PI3K in commitment to DNA synthesis, cell cycle status was determined at various times after stimulation. Measurement of DNA content indicated that some anti-IgM-stimulated cells initiated DNA replication (S phase) between 24 and 32 h, whereas LPS-treated cells began to enter S phase before 24 h (Fig. 2A, ethanol (EtOH)-treated controls). Inhibition of PI3K by pretreatment with LY294002 blocked progression of cells into S phase, although a small fraction of cells in S phase were detected at 48 h (Fig. 2A). LY294002 pretreatment modestly delayed S phase entry in cells stimulated with anti-CD40 plus IL-4.

Posttreatment of anti-IgM-stimulated cells at 16 h with LY294002 was able to effectively block the entry of virtually all cells into S phase (Fig. 2B). Twenty-four hours posttreatment with LY294002 did not affect the percentage of cells in S phase at 32 h but decreased ongoing DNA synthesis measured at 40 and 48 h (Fig. 2B). These data suggest that late PI3K-dependent signals are received between 16 and 24 h, during G<sub>1</sub> phase but before commitment to DNA synthesis. The reduced S phase at 40 or 48 h in 24-h treated cultures could be due to inhibition of successive cell divisions (see below).

Posttreatment of LPS-stimulated cells with LY294002 at 16 and 24 h inhibited the entry of cells into S phase as compared with the EtOH-treated controls (Fig. 2C). In both cases the percentage of cells in S phase was greater than in cultures pretreated with the drug, consistent with the differences seen in thymidine incorporation assays (Fig. 1A). Nevertheless, these data support a role for

PI3K signaling during cell cycle progression of B cells activated by LPS.

Activation of resting B cells is accompanied by a substantial increase in cell size (Fig. 2, D–F). Pretreatment with LY294002 markedly reduced this increase. In addition, the timing of later LY294002 additions correlated with an arrest in cell size increases. Specifically, reduced cell size became apparent 8–16 h after addition of the inhibitor. This effect of the inhibitor was most striking in cells stimulated with anti-IgM (Fig. 2D), but was also noticeable in LPS-stimulated cells (Fig. 2E). Pretreatment with LY294002 delayed but did not arrest cell size increases following stimulation with anti-CD40 plus IL-4 (Fig. 2F), consistent with the modest delay in S phase entry.

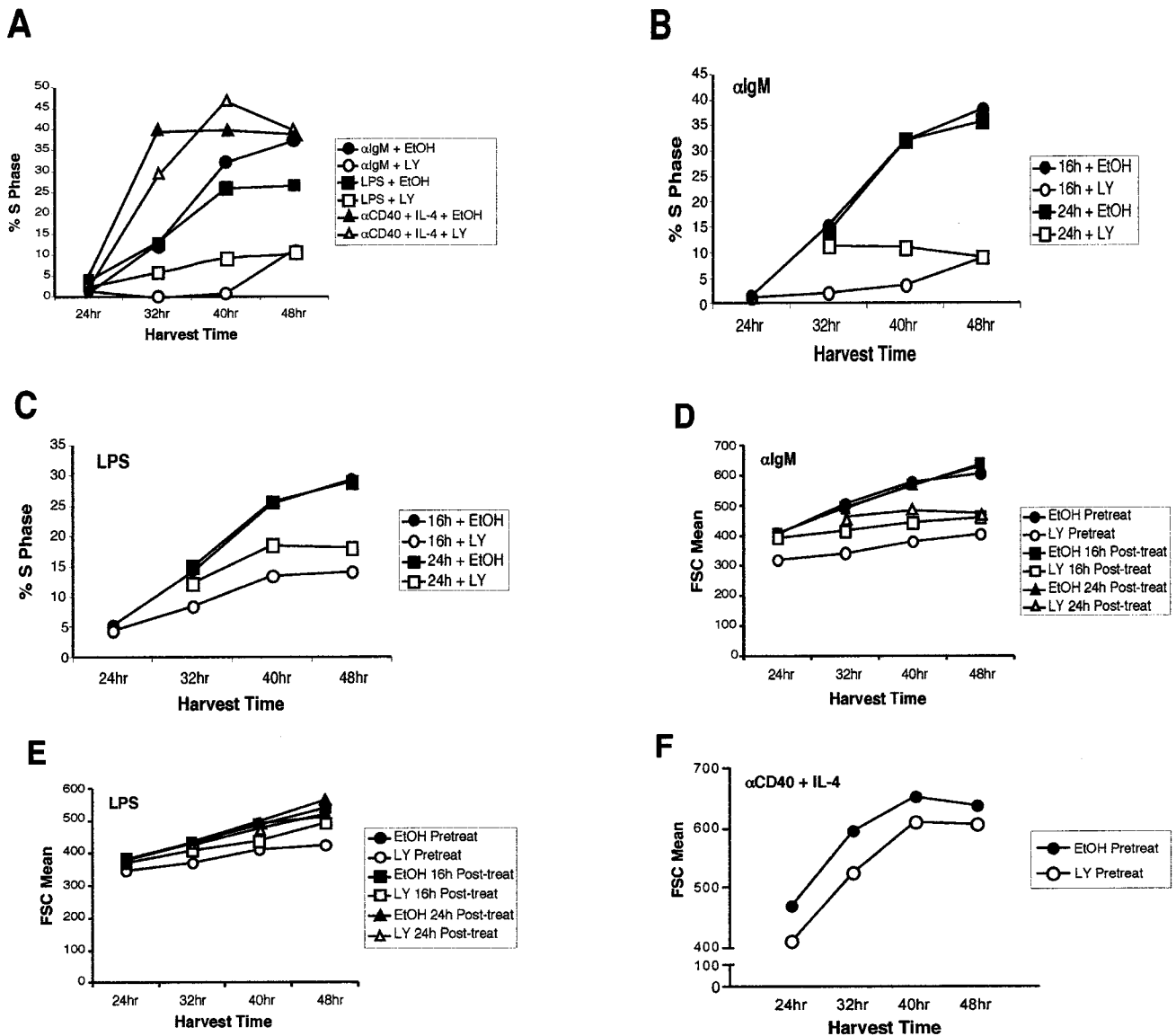
#### *Biochemical evidence of late PI3K signaling*

As a biochemical correlate of PI3K activation, we assessed the phosphorylation of p70S6kinase (S6K1) using a sensitive Ab that detects a mobility shift in the phosphorylated, activated enzyme. Phosphorylation and activation of S6K1 is dependent on PI3K activation; it is both directly phosphorylated by phosphoinositide-dependent kinase 1 and regulated by the Akt substrate Tuberin (20, 21). The activity of S6K1 has been shown to correlate with both protein synthesis and cell size (22).

S6K1 exhibits a characteristic ladder of bands when activated (Fig. 3). The fastest migrating band represents the dephosphorylated, least active form. Rapamycin is known to inhibit both basal and stimulated S6K1 phosphorylation. In agreement with published results (23), BCR engagement increased S6K1 phosphorylation at 5 min (our unpublished observations) and 1 h, and this was abrogated by pretreatment with the PI3K inhibitors LY294002 or wortmannin or by the mTOR inhibitor rapamycin (Fig. 3A). Increased S6K1 phosphorylation was maintained between 16 and 24 h (Fig. 3B). Addition of LY294002, wortmannin or rapamycin at 21.5 h blocked the phosphorylation measured at 22 or 23 h (Fig. 3C), demonstrating the continuing dependence on PI3K and mTOR function. We also observed sustained activation of S6K1 in cells activated with LPS (Fig. 3B). Interestingly, pretreatment with LY294002 blocked initial S6K1 activation (1 h) by anti-CD40 plus IL-4, but only partially reduced activation at 24 h (Fig. 3D). The extent of the S6K1 mobility shift in LY294002-treated samples at 24 h was similar to that seen in anti-IgM cultures pretreated with EtOH. Both early and late S6K1 activations were sensitive to rapamycin. This finding suggests a novel mechanism for S6K1 activation that is mTOR dependent but partially PI3K independent. Furthermore, the partial retention of S6K1 activation may explain why LY294002 delays but does not arrest cell size increases or S phase entry in B cells stimulated with anti-CD40 plus IL-4. The weak signal at 24 h in LY294002-pretreated cells stimulated with anti-IgM was consistently observed and probably is the result of reduced live cell recovery. On longer exposure, a single band comigrating with the rapamycin-pretreated sample was detected.

#### *Role of sustained Ag receptor signaling in cell cycle progression*

The extracellular signals that activate PI3K and S6K1 at late time points could be provided by an autocrine growth factor or by the original stimulus remaining in the culture. To test the role of continued BCR cross-linking during cell cycle progression, we used the Fab of anti-IgM as a competitive inhibitor. As expected, Fab were able to inhibit proliferation in a dose-dependent manner when added before the F(ab')<sub>2</sub> (Fig. 4A). Notably, partial inhibition was also observed when Fab were added at 16, 24, or 40 h poststimulation. In control experiments, the Fab did not deliver a proliferative signal when added to cells alone; furthermore, it did not



**FIGURE 2.** Inhibition of PI3K at late time points blocks cell cycle entry and cell size increases. B cells were stimulated with the indicated mitogens and cells were harvested at the times shown. Propidium iodide staining was used as a measure of cell DNA content. Cells were either pretreated (A) or posttreated at 16 or 24 h (B and C) with LY294002 or vehicle (EtOH). The percentage of cells in S phase was calculated using ModFit software. (D–F) Cell size as determined by mean forward scatter in a live cell gate for anti-IgM-stimulated (D), LPS-stimulated (E) cells, and anti-CD40 plus IL-4-stimulated (F) cells. Data shown are from one representative experiment of four. In other experiments, we determined the mean forward scatter of B cells to be  $\sim$ 330 at 0 h and 360 at 16 h with anti-IgM or LPS.

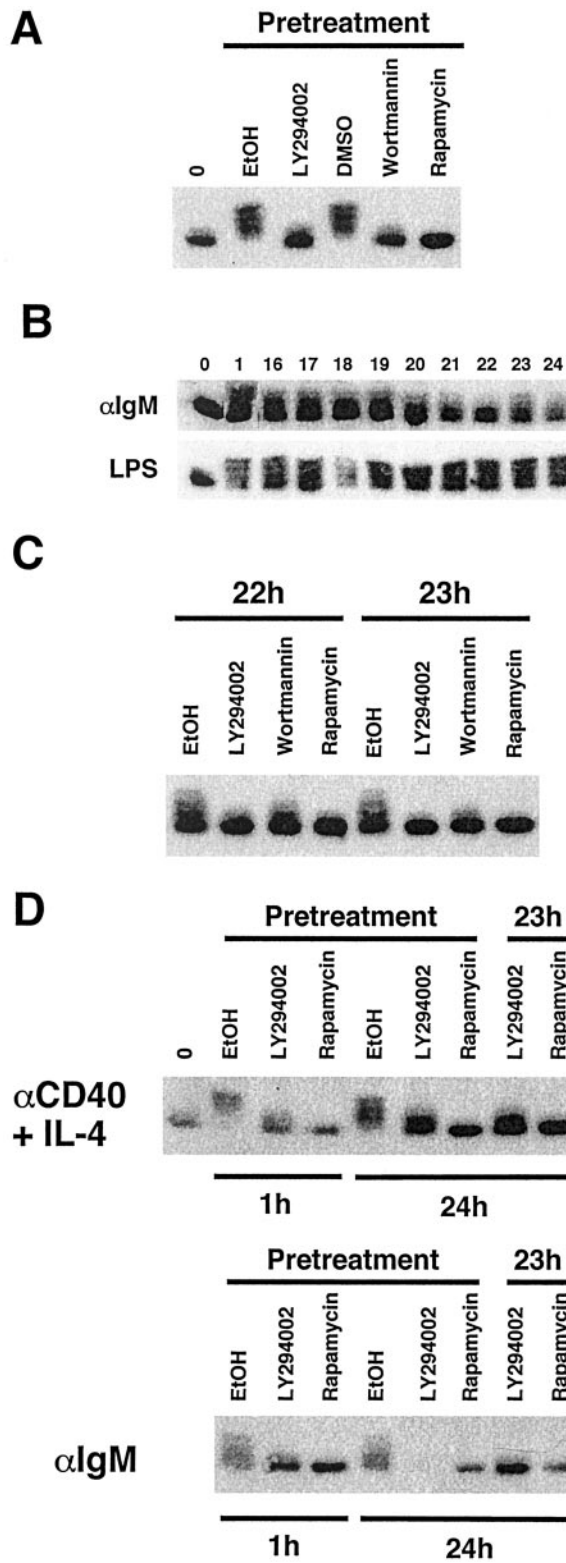
interfere with the proliferation of cells stimulated with LPS (our unpublished observations).

These results indicated that sustained or recurrent cross-linking of the BCR by anti-IgM F(ab')<sub>2</sub> is required for maximal cell cycle progression. Two groups reported in the early 1980s that washing out anti-IgM 24 h after stimulation prevents entry of B cells into S phase (4, 5). We performed similar washout experiments to confirm and extend these findings in our system. Cells were washed at various times (16, 24, and 40 h) after activation, placed back in culture with various stimuli or medium alone, and proliferation was measured by thymidine incorporation from 48 to 64 h (Fig. 4B). Up to 40 h of exposure to anti-IgM was not sufficient to sustain proliferation when cells were washed and placed into medium alone (only 18% of continuous stimulation). Addition of fresh anti-IgM restored proliferation, actually causing an increase relative to continuously treated cells. This is presumably attributable to increasing the effective concentration of mitogen relative to

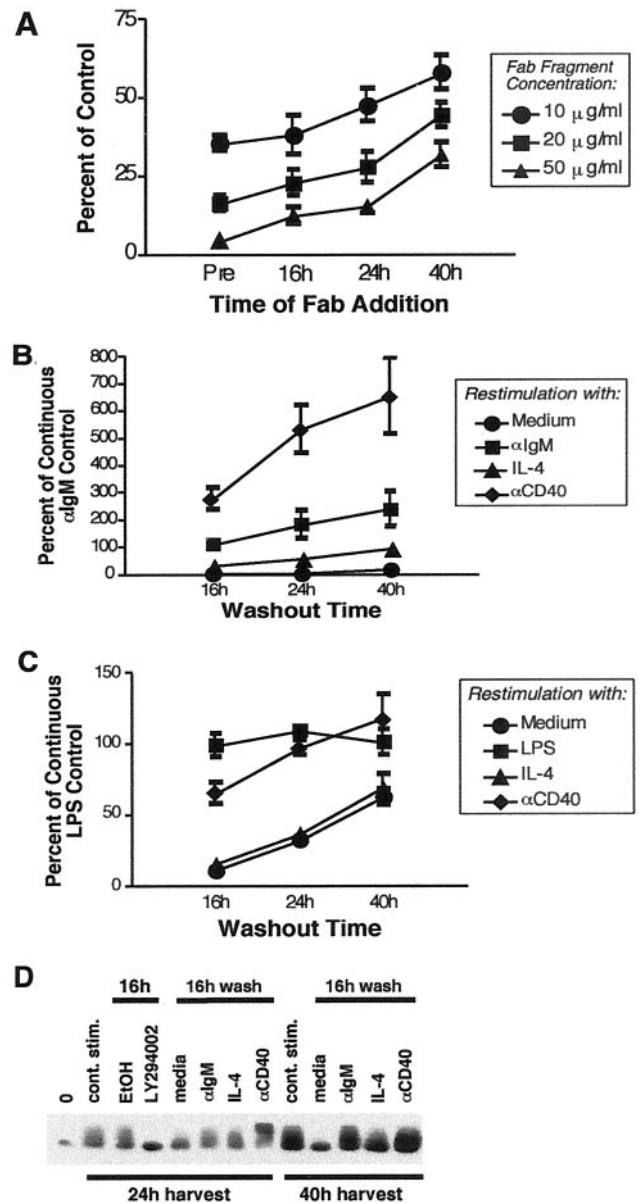
continuously growing cells, which may have internalized some anti-IgM. Together with the Fab data, these findings support a role for BCR signaling at time points after the initial activation event.

In B cells stimulated through the BCR, the receptor complex and bound Ab or specific Ag are internalized and delivered to a compartment for degradation and MHC loading (24). To support the notion of an additional late signal through the BCR, we sought evidence that IgM was expressed on the cell surface at later time points. Although levels of IgM dropped immediately following stimulation, there was always some IgM detected on the surface by FACS when compared with isotype control staining and by 40 h levels were beginning to rise once more (our unpublished observations).

The ability of T cell-derived signals to substitute for sustained BCR signaling was also tested. Addition of the cytokine IL-4 partially restored proliferation and was most effective when added after the 40-h wash. Strikingly, cells washed and replated with anti-CD40 led to proliferation levels of up to 7-fold higher than the



**FIGURE 3.** Biochemical evidence of late PI3K activation. B cells were stimulated with anti-IgM, harvested, lysed, and immunoblotted with anti-S6K1. *A*, Cells pretreated with the inhibitors shown or vehicle and harvested at 1 h. *B*, Cells were harvested at the indicated times (h) after stimulation with anti-IgM or LPS. *C*, Cells were stimulated with anti-IgM, treated with the indicated compounds or EtOH at 21.5 h, and then harvested at 22 or 23 h. *D*, Cells were pretreated with the inhibitors shown or vehicle and harvested at 1 or 24 h. Each experiment in this figure was repeated two to four times with comparable results. The increased intensity of the S6K1 signal consistently observed at later time points presumably reflects increased synthesis of cellular proteins as activated cells grow in size.



**FIGURE 4.** Optimal B cell proliferation and S6K1 activation requires the prolonged action of initial stimulus or T cell help. *A*, B cells were stimulated with the F(ab')<sub>2</sub> of anti-IgM and pre- or posttreated with increasing concentrations of Fab. Proliferation was measured by thymidine incorporation, with data expressed as percentage of untreated control. Shown is the mean ± SEM of four experiments. *B* and *C*, B cells were stimulated with anti-IgM (*B*) or LPS (*C*), and stimuli were removed by washing at the times shown. Cells were restimulated with the indicated mitogens or medium alone, and proliferation was measured by thymidine incorporation. Data are expressed as percentage of continuous stimulation (i.e., unwashed) control. Shown is the mean ± SEM of three experiments. *D*, S6K1 phosphorylation was detected by immunoblot in B cells stimulated with anti-IgM for the times indicated below. In the 16-h wash samples, the conditions of replating are indicated above. Data are representative of two experiments.

continuous stimulation control (Fig. 4*B*). Anti-CD40 alone induces modest proliferation that is comparable to anti-IgM alone (unpublished observations). These observations indicate that engagement of CD40, mimicking contact-dependent T cell help, is sufficient to provide late signals to drive proliferation of BCR-activated cells. The combination of anti-CD40 and IL-4 was not

tested in this system because this treatment is sufficient to drive maximal B cell proliferation without a BCR priming step.

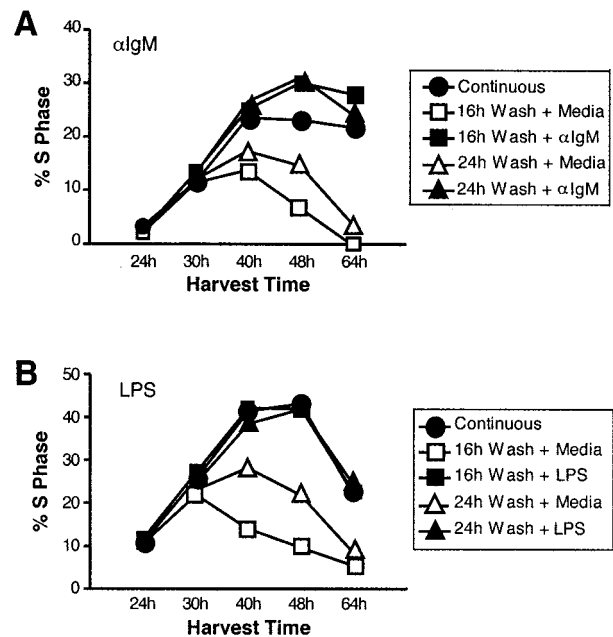
The status of S6K1 phosphorylation in washout experiments (Fig. 4D) correlated with the results seen in the thymidine incorporation assays. Specifically, cells washed at 16 h and placed back in medium alone showed less phosphorylation at 24 h compared with continuously treated cells, and virtually none at 40 h. Cells restimulated with anti-IgM showed slightly more phosphorylation than continuously stimulated cells, whereas cells restimulated with IL-4 showed slightly less at both time points. Restimulation with anti-CD40 enhanced S6K1 phosphorylation at both time points. These results show that the activation of S6K1, shown previously to be PI3K dependent, correlates with the continued presence of external mitogenic factors.

In the case of activation with LPS (Fig. 4C), optimal proliferation also depended on continued presence of the initial stimulus at 16 and 24 h. However, in contrast to the results with anti-IgM, removal of LPS at 40 h reduced DNA synthesis by only 50%. Restimulation with IL-4 alone had no effect and restimulation with LPS restored proliferation to control levels and no more. Again, restimulation at 16 or 24 h with anti-CD40 was able to restore proliferation as compared with medium, but here the effect was not as striking as with initial anti-IgM stimulation. This is probably because LPS stimulation induces maximal B cell proliferation (325,000 cpm, mean of three experiments.) that cannot be further augmented, whereas the response to anti-IgM is more limited (68,000 cpm,  $n = 3$ ). We attempted a separate approach to interfering with ongoing LPS responses by using a mAb to mouse TLR4. However, this reagent did not block LPS-driven proliferation even when added to cells before stimulation (our unpublished observations).

#### Role of sustained Ag receptor signaling in successive cell divisions

The thymidine incorporation assays indicated that washing out anti-IgM or LPS at 16 or 24 h markedly reduced DNA synthesis measured from 48 to 64 h. However, DNA content assays (Fig. 2A) showed that cells start to enter the first S phase before 32 h, well before this time window. To determine whether the continuous presence of the stimulus was required for commitment to the first S phase, we analyzed the cell cycle status of cells at various time points after washout or addition of Fab. We observed that blocking BCR function at 16 h had little effect on S phase entry at early time points (30 h) but reduced the percentage of cells in S phase at 40 h and even more at 48 or 64 h (Fig. 5A). Similarly, washing out LPS reduced the percentage of cells in S phase at later time points (Fig. 5B). These data suggest that sustained Ag receptor signaling in the first  $G_1$  phase is not required for the first cell division but promotes ongoing cell division. This distinction was masked when DNA synthesis was measured at 48 h in washout cultures (Refs. 4 and 5 and Fig. 4).

To study further the cell division and death patterns under different conditions, we labeled cells with CFSE before initiation of culture. Fig. 6A documents the mitotic history of cells continuously treated with anti-IgM or LPS. Histograms of the CFSE content of live cells (gate 1, left panel, Fig. 6E) showed that few if any cells had divided once by 24 h following treatment with anti-IgM or LPS. With anti-IgM, many cells had divided once by 40–48 h and twice by 64 h; a few had completed a third division by 64 h. A high percentage of apoptotic cells following anti-IgM stimulation had been noted previously (2, 3), but the cell division history of the dying cells was not addressed. Using annexin V as a marker of apoptosis, we observed considerable death among both undivided and divided cells following 64 h of continuous anti-IgM



**FIGURE 5.** Sustained Ag receptor engagement promotes ongoing cell division, but is not required for initial S phase entry. B cells were stimulated with anti-IgM (A) or LPS (B), washed at 16 or 24 h, and placed back in culture with medium or mitogen. Cells were harvested at the indicated times, fixed, and stained with propidium iodide. Percent S phase was determined as in Fig. 2. A representative experiment of three is shown.

(Fig. 6B, left panel). In cultures treated with LPS, it was evident that many cells had completed one or two divisions by 40 h and four or five divisions by 64 h (Fig. 6A). The fraction of dying cells was substantially lower in LPS-treated cultures than in anti-IgM cultures (Fig. 6B, right panel).

The effect of other cell manipulations was assessed after 64 h of total culture time. Washing out anti-IgM at 16 h did not prevent the first cell division, compared with cells with continuously stimulated or restimulated with anti-IgM (Fig. 6B). However, the washout did cause most of the divided cells to die rather than to complete further divisions. A few cells appeared to complete two divisions before dying. Similarly, washing out LPS after 16 h did not prevent initial cell division but greatly reduced the accumulation of live cells in later divisions (Fig. 6B). Washing out anti-IgM or LPS after 24 h yielded similar although less dramatic decreases in recovery of live cells that had completed more divisions (Fig. 6B). A higher concentration of anti-IgM (50  $\mu$ g/ml) increased the extent of cell division, but did not prevent death, either in continuous stimulation or washout conditions (our unpublished observations).

As noted above, thymidine incorporation measured 48–64 h after anti-IgM treatment was still reduced by treatment at 40 h with PI3K inhibitors, the Fab, or removal of stimulus. Since these cells initiate S phase between 24 and 30 h and have completed one division by 40 h, this suggests that BCR signaling and PI3K activation are still required in daughter cells undergoing successive cell divisions. This idea was supported by CFSE data. When cells were washed at 40 h and replated in medium without mitogen, analysis at 64 h showed fewer cells having completed three or four divisions (Fig. 6B). A substantial increase in apoptosis was also observed (Fig. 6B). Similar results were seen when Fab of anti-IgM were added at 40 h (our unpublished observations). Overlays of histograms of live cells clearly show the effects of washing out anti-IgM at 16, 24, or 40 h (Fig. 6C). For cells washed at 40 h and

restimulated with anti-IgM, it appeared that they had not undergone many more divisions than those stimulated continuously, but that there was noticeably less death (Fig. 6, *B* and *C*).

To determine the minimum duration of Ag receptor stimulation required for B cells to commit to initial and subsequent cell divisions, we stimulated CFSE-labeled cells and washed out the stimulus at time points ranging from 1 to 6 h. The results showed that 1 h of exposure to anti-IgM was sufficient to trigger one round of cell division in a fraction of the cells (Fig. 6*D*; compare to medium alone, Fig. 6*B*); however, nearly all of the divided cells died without completing more divisions. Increasing numbers of divided, dying cells were observed following 2-, 4-, or 6-h exposure times (Fig. 6*D* and our unpublished observations). Together with the 16- and 24-h washout data, these findings suggest that exposure to anti-IgM for >6 h is absolutely required for B cells to complete multiple cell divisions. We did not determine whether the fraction of cells that divide once following brief BCR engagement represent resting follicular B cells or a minority subset of splenic B cells with distinct developmental properties.

LPS-treated cells showed different behavior than anti-IgM-treated cells in washout experiments. 1–6 h of exposure to LPS triggered a smaller fraction of cells to divide once (Fig. 6*D*). In contrast, by 16 h, most LPS-treated cells had committed to one or two divisions (Fig. 6*B*). Compared with continuously treated cells or restimulated cells, there was more death and fewer cells showing several divisions (overlays, Fig. 6*C*). Progressively smaller effects were seen at later washout times (Fig. 6, *B* and *C*) in keeping with the results of thymidine incorporation experiments.

#### *Role of PI3K in successive cell divisions*

When CFSE-labeled cells were pretreated with LY294002 before anti-IgM treatment, few live cells remained at 64 h, and these were dying without ever having divided (Fig. 7*A*). Treatment with the PI3K inhibitor at 24 h resulted in the death of most of the cells by 64 h, but it was clear that some had entered the cell cycle before drug treatment and completed at least one round of division before dying (Fig. 7*A*). Treatment with LY294002 at 40 h also caused a great deal of cell death, although many had progressed as far as two rounds of division before dying (Fig. 7*A*). It was apparent that inhibition of PI3K caused much more death than simple removal of BCR stimulus.

Pretreatment with LY294002 increased the percentage of undivided cells following LPS treatment and reduced the fraction of cells completing multiple divisions (Fig. 7, *B* and *C*). Interestingly, pretreatment with the drug did not cause as much death as in cultures stimulated with anti-IgM, suggesting that LPS provides a survival signal that is PI3K independent and distinct from the proliferative signal. Addition of LY294002 at 24 or 40 h had fewer effects, mainly reducing the fraction of cells undergoing the last division (Fig. 7, *B* and *C*). In general, the effects of LY294002 on LPS-treated cells were less dramatic when assayed by CFSE (Fig. 7, *B* and *C*) than by thymidine incorporation (Fig. 1*A*), presumably because the thymidine pulse from 48 to 64 h reads out S phase activity at a time corresponding to the last few divisions, whereas CFSE measures the entire division history.

## **Discussion**

We have demonstrated that sustained or recurrent receptor engagement is necessary for maximal proliferation and survival of B cells activated via either BCR cross-linking or LPS. In the case of anti-IgM, as little as 1 h of exposure is sufficient to trigger a single cell division in a fraction of the population; however, all of the daughter cells die. The preparation of purified B cells used in this study contains all splenic subsets (transitional, follicular, and marginal

zone); thus, it is possible that the fraction of cells that do complete a single division represent a minor developmental subset rather than a portion of the mature recirculating B cell pool. As much as 24 h of exposure to anti-IgM, a time at which cells are beginning to enter S phase, is not enough to sustain survival during multiple cell divisions. Strikingly, a BCR signal is still required for ongoing cell division in daughter cells, as illustrated by the reduced survival in cells washed or treated with Fab at 40 h. Thus, commitment to the cell cycle does not ensure survival. Even with continuous BCR engagement, expansion of B cells is limited by a high degree of cell death in daughter cells. Replenishing the supply of anti-IgM partially restores survival. Addition of T cell-derived help in the form of IL-4 or anti-CD40 partially or completely replaces the requirement for prolonged exposure to stimulus.

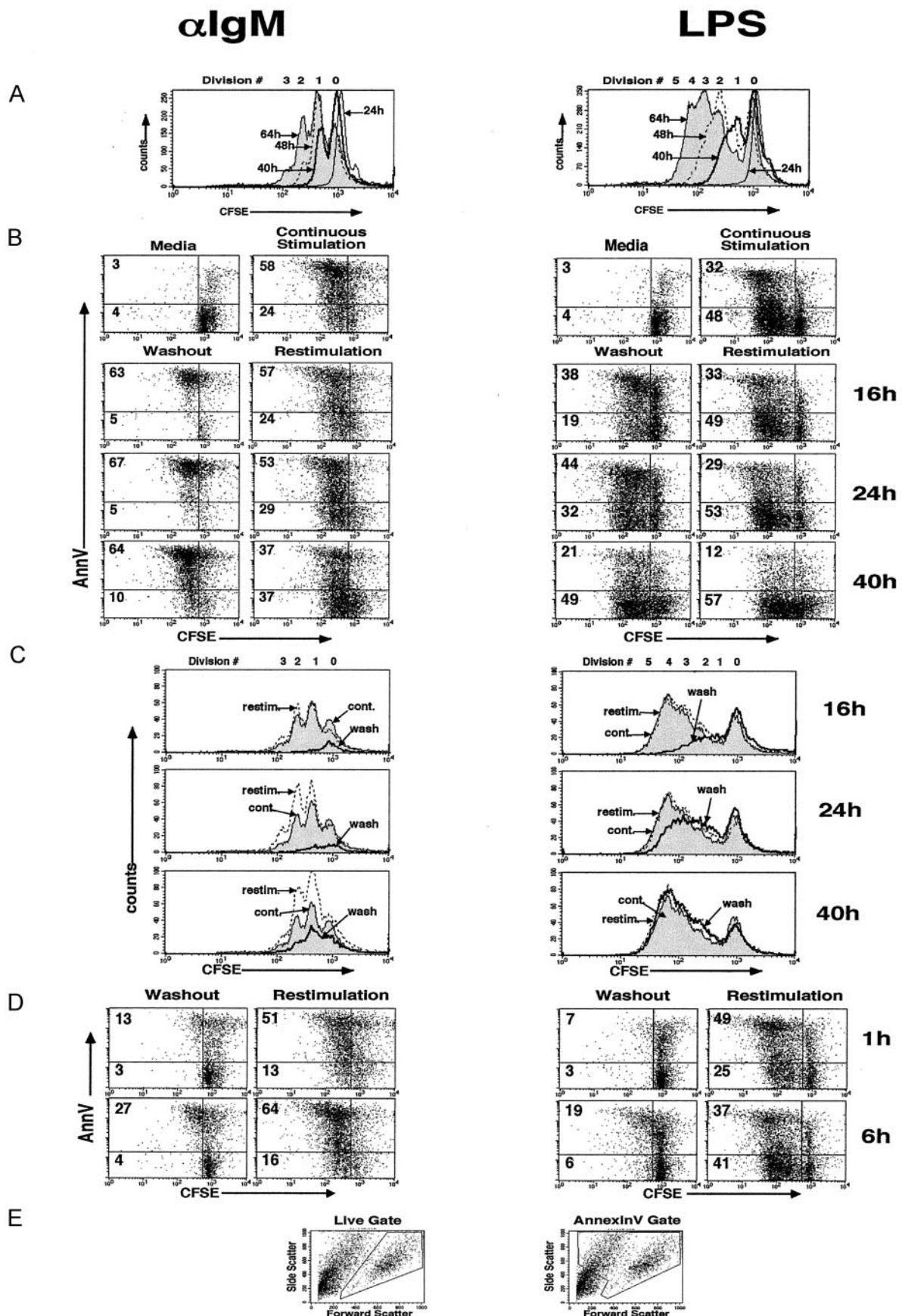
The use of CFSE in these experiments extended previous findings concerning the duration of BCR engagement required for proliferation. Using thymidine incorporation at 48 h as a measure of cell cycle progression, two groups found that most B cells required anti-IgM to be present for >24 h (4, 5). Furthermore, less than 12 h of stimulation yielded no apparent proliferation. It should be noted that B cells that recognize Ag *in vivo* without T cell help may nonetheless receive additional survival signals (e.g., BAFF) not present in the *in vitro* culture systems used in the current and previous studies.

Our findings in B cells are similar to observations of Ag-specific CD4<sup>+</sup> T cells, in that brief exposure to Ag was able to trigger cell division but optimal survival required sustained TCR engagement (8, 9). However, a significant fraction of those T cells survived to complete multiple cell divisions after 2 h of Ag exposure, whereas 2 h of exposure to anti-IgM led to the death of nearly all B cells after one division. In contrast to B cells and CD4<sup>+</sup> T cells, maximal survival and expansion of CD8<sup>+</sup> T cells appear to proceed with as little as 2 h of Ag receptor engagement (6, 7). In comparing our results to those obtained in T cells, it is important to keep in mind that T cell stimulation was achieved using APC, leading to engagement of additional receptors besides the TCR. When CD4<sup>+</sup> T cells were stimulated with purified MHC-peptide complexes, conditions more similar to anti-IgM treatment of B cells, at least 20 h of exposure was necessary to trigger measurable thymidine incorporation (25).

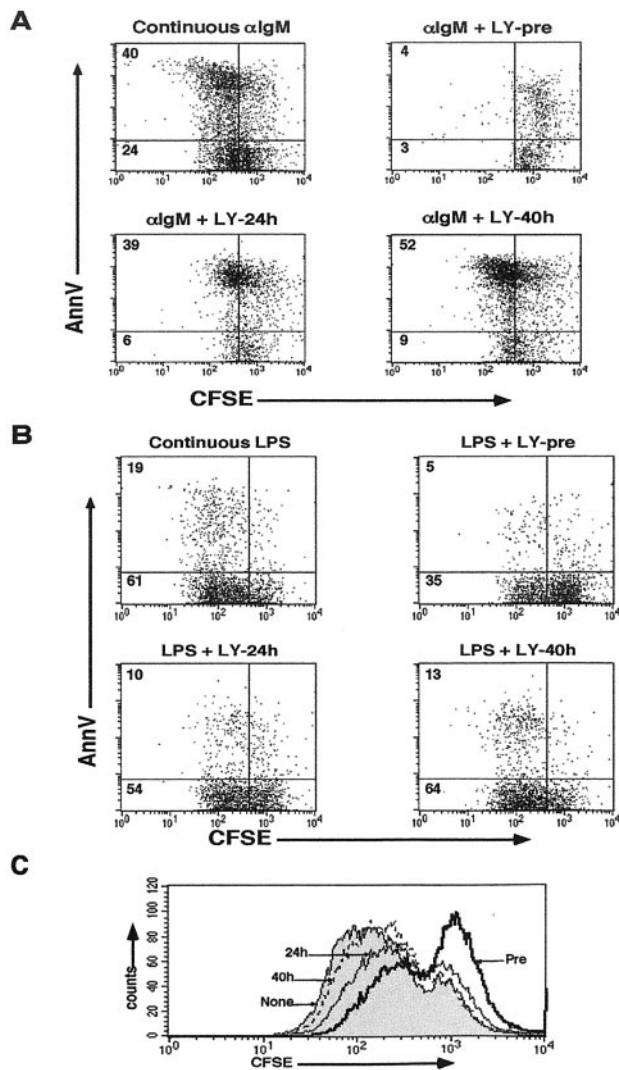
Pretreatment with PI3K inhibitors reduces BCR-dependent calcium mobilization (26) as well as phosphorylation of Akt and S6K1 (Fig. 3 and Refs. 23, 27, and 28), documenting a required role for PI3K activation in early cellular responses. Our data provide additional evidence that PI3K is activated and has a required function at later stages of B cell activation. Analysis of S6K1 phosphorylation and its sensitivity to PI3K inhibitors indicated that PI3K activation is maintained between 16 and 24 h after treatment with anti-IgM or LPS (Fig. 3). Phosphorylation of Akt is another response often measured as a biochemical readout of PI3K activation. As reported by others (27, 28), we observed increased Akt phosphorylation within 5 min after BCR cross-linking, and this was blocked by LY294002 pretreatment (our unpublished observations). Although we also detected Akt phosphorylation 16 or 24 h following stimulation, the stoichiometry was lower and surprisingly could not be reduced by treatment with LY294002 (our unpublished observations), in contrast to S6K1 phosphorylation. This suggests that the signaling pathway leading from PI3K lipid products to S6K1 activation differs at early and late time points, a hypothesis we are currently testing further.

Several observations show the functional role of PI3K during cell cycle progression and expansion of B cells. Addition of PI3K inhibitors 16 h after anti-IgM prevents entry of cells into the S phase. Addition of the inhibitors at 24 or 40 h reduces thymidine





**FIGURE 6.** Survival during successive cell divisions requires prolonged exposure to anti-IgM (left panels) or LPS (right panels). B cells were labeled with CFSE before carrying out stimulation/washout experiments. **A**, The extent of cell division was analyzed in continuously stimulated cells harvested at the indicated times (arrows). Only cells in a live gate based on forward and side scatter (**E**, left panel) are included in the histograms. Flow cytometry settings were adjusted so that undivided cells (based on unstimulated controls) formed a peak around 1000 relative fluorescence units (Figure legend continues)



**FIGURE 7.** PI3K inhibitor has differential effects on cell division and death in B cells stimulated with anti-IgM vs LPS. B cells labeled with CFSE and stimulated with the anti-IgM (A) or LPS (B and C) were pre- or posttreated with LY294002 at the indicated times, harvested at 64 h, and stained with AnnexinV<sup>PE</sup>. Dot plots and quadrant numbers in A and B, and histograms in C, were generated as described in the legend to Fig. 6. A representative experiment of three is shown.

incorporation from 48 to 64 h, reduces the fraction of cells in S phase during this time window, and results in the death of cells after one or two cell divisions. The effects of LY294002 on LPS-treated cultures were similar to those of anti-IgM cultures, although later drug treatments had a lesser effect. Thus, once LPS-activated B cells have completed one division, further proliferation and survival is partially independent of PI3K function. In addition, pretreatment with the drug mainly reduced the percentage of cells completing cell divisions without greatly increasing the fraction of dying cells. These findings suggest either that LPS-mediated sur-

vival signals do not require PI3K or that residual macrophages are very active in phagocytosing apoptotic cells when exposed to LPS.

Distinct waves of PI3K activation have also been observed in fibroblasts, the second occurring during the G<sub>1</sub> phase several hours after addition of the growth factor (29). Two recent articles used fluorescence microscopy to demonstrate elegantly that Ag-specific T cell activation is accompanied by sustained production of the PI3K lipid product phosphatidylinositol 3,4,5-triphosphate (16, 30). However, the role of PI3K in Ag receptor-stimulated primary T and B cells differs in at least three respects. First, acute calcium mobilization is blocked by PI3K inhibitors in B cells (26) but not in T cells (30). Second, up-regulation of the activation marker CD25 is blocked by LY294002 in B cells (our unpublished observations) but not in T cells (16). Third, LY294002 no longer blocks T cell proliferation when added 24 h after Ag exposure (16), a time point at which this drug still attenuates anti-IgM or LPS-driven B cell proliferation.

The signals for B cell proliferation and survival provided by sustained Ag receptor signaling are probably supplemented by additional autocrine factors or serum components that activate PI3K. This was suggested by the greatly increased death observed following treatment with LY294002 at 24 or 40 h compared with removal of stimulus at these time points. Similarly, addition of LY294002 at 16 h completely prevented S phase entry whereas washout of anti-IgM at 16 h allowed the first division to proceed.

In addition to the observed survival signal provided by PI3K, our data document a role for PI3K in B cell growth (size increase). The pathway leading from PI3K to S6K1 plays an evolutionarily conserved role in regulation of protein synthesis and cell size (22). Addition of LY294002 rapidly inactivated S6K1 and blunted the ongoing increase in cell size that is characteristic of activated lymphoid blasts. It is possible that the failure to commit to DNA synthesis in cells treated with LY294002 at 16 h is the result of a G<sub>1</sub> phase block at a cell size checkpoint. In addition, it is worth considering that apoptosis in daughter cells following LY294002 treatment or washout of stimulus at 24 or 40 h may occur because the parental cell cannot grow to a sufficient size and synthesize enough protein to allow for the multiple rapid cell divisions that normally occur during clonal expansion. Accordingly, we found that the status of S6K1 phosphorylation correlated with the proliferative capacity of B cells in washout experiments. The importance of this pathway is underscored by the finding that rapamycin, whose inhibition of mTOR leads to rapid inactivation of S6K1, also diminished B cell proliferation when added at late time points. Furthermore, the inability of LY294002 to block proliferation driven by anti-CD40 plus IL-4 is associated with activation of S6K1 that is partially LY294002 resistant.

In summary, our findings suggest that the outcome of an encounter with a TI Ag depends upon the duration of the interaction. Brief exposure to Ag in the absence of T cell help is insufficient to trigger significant clonal expansion. This suggests that naive B cells are programmed to ignore transient exposure to Ags that might be rapidly cleared by the innate immune system or by memory B cell responses. An important biochemical correlate of clonal

(note log scale). The shoulder to the right of this peak was also seen in the unstimulated cells and may represent aggregates. The positions of peaks corresponding to cell division numbers are shown above. B and D, Cell division history and cell death was compared in B cells subject to continuous stimulation, washout at the indicated times, or washout and restimulation. Cells were collected at 64 h, stained with AnnexinV<sup>PE</sup>, and analyzed by flow cytometry using the gate shown in E, right panel. Quadrants were drawn based on the pattern in unstimulated cells. The percentages of live divided cells and dying divided cells are indicated in the lower and upper left quadrants. C, Analysis of CFSE fluorescence from 16-, 24-, and 40-h washout experiments using a live cell gate. The histograms corresponding to continuous (cont.), washout (wash), and restimulation (restim.) are indicated. E, Dot plots of forward and side scatter from a stimulated sample illustrating the two gates used for the different analyses. Left panel, live cell gate; right panel, live plus dying cell gate. All data shown are from a representative experiment of five.

expansion is the activation of PI3K and S6K1. It is noteworthy that blockade of PI3K signaling plays a role in a distinct mechanism that limits ongoing B cell responses, namely, the engagement of the inhibitory receptor Fc $\gamma$ RIIb by IgG-coated Ags (27, 31). Thus, PI3K signaling is a common control point for B cell fate decisions.

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## References

- Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1:135.
- Anderson, J. S., M. Teutsch, Z. Dong, and H. H. Wortis. 1996. An essential role for Bruton's tyrosine kinase in the regulation of B-cell apoptosis. *Proc. Natl. Acad. Sci. USA* 93:10966.
- Fruman, D. A., S. B. Snapper, C. M. Yballe, L. Davidson, J. Y. Yu, F. W. Alt, and L. C. Cantley. 1999. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85 $\alpha$ . *Science* 283:393.
- Sieckmann, D. G. 1980. The use of anti-immunoglobulins to induce a signal for cell division in B lymphocytes via their membrane IgM and IgD. *Immunol. Rev.* 52:181.
- DeFranco, A. L., E. S. Raveche, and W. E. Paul. 1985. Separate control of B lymphocyte early activation and proliferation in response to anti-IgM antibodies. *J. Immunol.* 135:87.
- Kaech, S. M., and R. Ahmed. 2001. Memory CD8<sup>+</sup> T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat. Immunol.* 2:415.
- van Stipdonk, M. J., E. E. Lemmens, and S. P. Schoenberger. 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat. Immunol.* 2:423.
- Lee, W. T., G. Pasos, L. Cecchini, and J. N. Mittler. 2002. Continued antigen stimulation is not required during CD4<sup>+</sup> T cell clonal expansion. *J. Immunol.* 168:1682.
- Lee, K. H., A. D. Holdorf, M. L. Dustin, A. C. Chan, P. M. Allen, and A. S. Shaw. 2002. T cell receptor signaling precedes immunological synapse formation. *Science* 295:1539.
- Hasbold, J., A. V. Gett, J. S. Rush, E. Deenick, D. Avery, J. Jun, and P. D. Hodgkin. 1999. Quantitative analysis of lymphocyte differentiation and proliferation in vitro using carboxyfluorescein diacetate succinimidyl ester. *Immunol. Cell Biol.* 77:516.
- Fruman, D. A., and L. C. Cantley. 2002. Phosphoinositide 3-kinase in immunological systems. *Semin. Immunol.* 14:7.
- Gold, M. R., and R. Aebersold. 1994. Both phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase products are increased by antigen receptor signaling in B cells. *J. Immunol.* 152:42.
- Bone, H., and N. A. Williams. 2001. Antigen-receptor cross-linking and lipopolysaccharide trigger distinct phosphoinositide 3-kinase-dependent pathways to NF- $\kappa$ B activation in primary B cells. *Int. Immunol.* 13:807.
- Bijsterbosch, M. K., C. J. Meade, G. A. Turner, and G. G. Klaus. 1985. B lymphocyte receptors and polyphosphoinositide degradation. *Cell* 41:999.
- Brennan, P., J. W. Babbage, B. M. Burgering, B. Groner, K. Reif, and D. A. Cantrell. 1997. Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* 7:679.
- Costello, P. S., M. Gallagher, and D. A. Cantrell. 2002. Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. *Nat. Immunol.* 3:1082.
- Suzuki, H., Y. Terauchi, M. Fujiwara, S. Aizawa, Y. Yazaki, T. Kadowaki, and S. Koyasu. 1999. Xid-like immunodeficiency in mice with disruption of the p85 $\alpha$  subunit of phosphoinositide 3-kinase. *Science* 283:390.
- Venkataraman, C., G. Shankar, G. Sen, and S. Bondada. 1999. Bacterial lipopolysaccharide induced B cell activation is mediated via a phosphatidylinositol 3-kinase dependent signaling pathway. *Immunol. Lett.* 69:233.
- Wicker, L. S., R. C. Boltz, Jr., V. Matt, E. A. Nichols, L. B. Peterson, and N. H. Sigal. 1990. Suppression of B cell activation by cyclosporin A, FK506 and rapamycin. *Eur. J. Immunol.* 20:2277.
- Alessi, D. R., M. T. Kozlowski, Q. P. Weng, N. Morrice, and J. Avruch. 1998. 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. *Curr. Biol.* 8:69.
- Manning, B. D., A. R. Tee, M. N. Logsdon, J. Blenis, and L. C. Cantley. 2002. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberlin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol. Cell* 10:151.
- Kozma, S. C., and G. Thomas. 2002. Regulation of cell size in growth, development and human disease: PI3K, PKB and S6K. *BioEssays* 24:65.
- Li, H. L., W. Davis, and E. Pure. 1999. Suboptimal cross-linking of antigen receptor induces Syk-dependent activation of p70S6 kinase through protein kinase C and phosphoinositol 3-kinase. *J. Biol. Chem.* 274:9812.
- Janeway, C. A., P. Travers, M. Walport, and M. J. Schlomchik. 2001. *Immunobiology: The Immune System in Health and Disease*. Garland, New York.
- Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 8:89.
- Buhl, A. M., C. M. Pleiman, R. C. Rickert, and J. C. Cambier. 1997. Qualitative regulation of B cell antigen receptor signaling by CD19: selective requirement for PI3-kinase activation, inositol-1,4,5- trisphosphate production and Ca<sup>2+</sup> mobilization. *J. Exp. Med.* 186:1897.
- Aman, M. J., T. D. Lamkin, H. Okada, T. Kurosaki, and K. S. Ravichandran. 1998. The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J. Biol. Chem.* 273:33922.
- Gold, M. R., M. P. Scheid, L. Santos, M. Dang-Lawson, R. A. Roth, L. Matsuuchi, V. Duronio, and D. L. Krebs. 1999. The B cell antigen receptor activates the Akt (protein kinase B)/glycogen synthase kinase-3 signaling pathway via phosphatidylinositol 3-kinase. *J. Immunol.* 163:1894.
- Jones, S. M., R. Klinghoffer, G. D. Prestwich, A. Tokar, and A. Kazlauskas. 1999. PDGF induces an early and a late wave of PI 3-kinase activity, and only the late wave is required for progression through G<sub>1</sub>. *Curr. Biol.* 9:512.
- Harriague, J., and G. Bismuth. 2002. Imaging antigen-induced PI3K activation in T cells. *Nat. Immunol.* 3:1090.
- Ono, M., S. Bolland, P. Tempst, and J. V. Ravetch. 1996. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc $\gamma$ RIIB. *Nature* 383:263.