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# Enhanced T Cell Proliferation in Mice Lacking the p85 $\beta$ Subunit of Phosphoinositide 3-Kinase<sup>1</sup>

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**Phosphoinositide 3-kinase activation is important for lymphocyte proliferation and survival. Disrupting the gene that encodes the major phosphoinositide 3-kinase regulatory isoform p85 $\alpha$  impairs B cell development and proliferation. However, T cell functions are intact in the absence of p85 $\alpha$ . In this study, we test the hypothesis that the related isoform p85 $\beta$  is an essential regulatory subunit for T cell signaling. Unexpectedly, T cells lacking p85 $\beta$  showed a marked increase in proliferation and decreased death when stimulated with anti-CD3 plus IL-2. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells completed more cell divisions. Transcriptional profiling revealed reduced levels of caspase-6 mRNA in p85 $\beta$ -deficient T cells, which was paralleled by reduced caspase-6 enzyme activity. Increased T cell accumulation was also observed in vivo following infection of p85 $\beta$ -deficient mice with mouse hepatitis virus. Together, these results suggest a unique role for p85 $\beta$  in limiting T cell expansion. *The Journal of Immunology*, 2004, 172: 6615–6625.**

**P**hosphoinositide 3-kinases (PI3Ks)<sup>3</sup> phosphorylate inositol phospholipids, thereby promoting membrane association of certain cytoplasmic proteins that specifically bind to PI3K lipid products (1–3). PI3K signaling promotes proliferation and survival in many cell types, both normal and transformed (3, 4). Cellular levels of PI3K products increase following engagement of the B cell receptor (BCR) and the TCR (5, 6). Elegant microscopy studies from two groups recently demonstrated that the PI3K lipid product phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>) accumulates at the T cell plasma membrane and is concentrated at the APC contact zone in an Ag-dependent manner (7, 8). Inhibitors of PI3K enzyme activity (wortmannin, LY294002) block Ag receptor-mediated proliferation of both T and B cells, illustrating the importance of PI3K signaling (9–12). Inhibitor studies also have demonstrated a role for PI3K in cytokine-driven proliferation and survival of lymphocytes (2, 11, 13). PI3K appears to regulate both shared and distinct pathways in T and B cells. For example, activation of Akt is PI3K dependent in both cell types (14), whereas calcium mobilization is more dependent on PI3K in B cells than in T cells (8, 15).

There are four subgroups of PI3K with distinct functions in cells (1, 3, 16). Class I<sub>A</sub> PI3Ks, which function downstream of activated tyrosine kinases, are heterodimers composed of a catalytic subunit and a tightly associated regulatory subunit. The regulatory subunit

influences both the activity and localization of the catalytic subunit. There are three known genes for each component of the heterodimer. The *Pik3ca*, *Pik3cb*, and *Pik3cd* genes encode the catalytic subunits p110 $\alpha$ , p110 $\beta$ , and p110 $\delta$ . The *Pik3r1*, *Pik3r2*, and *Pik3r3* genes encode regulatory subunits p85 $\alpha$ , p85 $\beta$ , and p55 $\gamma$ . Two additional products of the *Pik3r1* gene encode the proteins p55 $\alpha$  and p50 $\alpha$ . The different regulatory subunits can associate with the catalytic subunits interchangeably, but have unique tissue distributions. Each regulatory subunit possesses two highly conserved Src homology 2 (SH2) domains and a p110-binding domain in the C-terminal portion. The smaller isoforms possess short N-terminal segments before the first SH2 domain. The N-terminal portions of p85 $\alpha$  and p85 $\beta$  have a similar set of domains (SH3, RhoGAP homology, proline rich), but less primary sequence identity, suggesting that this region may mediate distinct functions.

Lymphocytes express each of the catalytic isoforms as well as the regulatory isoforms p85 $\alpha$  and p85 $\beta$  (11, 14). T cells also express p50 $\alpha$ . Genetic studies in mice have begun to elucidate the specific function of class I<sub>A</sub> PI3K isoforms in lymphocytes. Mice lacking only p85 $\alpha$  or lacking all variants of *Pik3r1* (p85 $\alpha$ , p55 $\alpha$ , and p50 $\alpha$ ) show comparable immune phenotypes (11, 17). Specifically, B cell development and proliferation are impaired, whereas T cell development and function are apparently normal. In contrast, a functional p110 $\delta$  catalytic subunit is required for optimal proliferation of both T and B cells (14, 18, 19). The demonstration that T cells require class I<sub>A</sub> PI3K signaling, but not p85 $\alpha$  or its variants, suggested that p85 $\beta$  may be the essential class I<sub>A</sub> regulatory subunit for T cell proliferation and development. In this study, we test this hypothesis by analyzing lymphocyte development and proliferation in mice lacking p85 $\beta$ . We show that B cell development and function in p85 $\beta$  knockout mice are indistinguishable from wild type. In contrast, we observe a surprising enhancement of proliferation in p85 $\beta$ -deficient T cells in vitro and a sustained expansion of T cells in vivo following viral infection.

## Materials and Methods

### Mice

The generation of mice lacking p85 $\beta$  (*Pik3r2* null) was described in detail elsewhere (20). Mice were maintained in a mixed background (C57BL/6  $\times$  129SvEv) for the experiments shown in this work and were studied at 2–4

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<sup>3</sup> Abbreviations used in this paper: PI3K, phosphoinositide 3-kinase; AICD, activation-induced cell death; BCR, B cell receptor; MHV, mouse hepatitis virus; NP, nitrophenol; PIP<sub>3</sub>, phosphatidylinositol-3,4,5-trisphosphate; PLC, phospholipase C; SH, Src homology.

mo of age. All procedures were approved by the institutional animal care and use committee.

### FACS analysis

Single cell suspensions of RBC-depleted spleens, peritoneal lavages, lymph nodes, thymus, or purified B and T cells from 6- to 12-wk-old mice were stained with different combinations of the following Abs: anti-IgD FITC, anti-B220 CyChrome, anti-CD5 FITC, anti-IgM PE, anti-CD8 PerCP, anti-Thy-1.2 PE (BD Biosciences, San Diego, CA), anti-CD62L PE, anti-CD25 PE, anti-CD44 FITC, anti-CD69 FITC, and anti-CD4 allophycocyanin (Ebioscience, San Diego, CA). For cell cycle analysis, DNA content was determined by staining fixed cells with propidium iodide (Roche, Nutley, NJ), as described (11). Cell divisions were tracked following staining of cells with CFSE (Molecular Probes, Eugene, OR). Cells were also stained with annexin V-PE (Caltag Laboratories, Burlingame, CA). Samples were acquired on a FACSCalibur (BD Biosciences), and data were analyzed by CellQuest (BD Biosciences) and FlowJo (Tree Star, San Carlos, CA) software. Modfit (Verity Software, Topsham, ME) software was used to quantitate cell cycle and apoptosis using the fluorescence values of the FL2-area channel.

### Immunoblotting

Thymocytes ( $5 \times 10^6$  for direct blotting) were lysed in a detergent buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 10% glycerol, 150 mM NaCl) containing a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO), and proteins were resolved by 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were then blocked in 5% milk in TBS for 1 h. Primary Abs used were purified rabbit antisera specific for p110 $\beta$  (H-198; Santa Cruz Biotechnology, Santa Cruz, CA), and p110 $\delta$  (kindly provided by B. Vanhaesebroeck, Ludwig Institute for Cancer Research, London, U.K.), a mixture of hybridoma supernatants specific for p110 $\alpha$  (clones U3A and 11A; kind gift of A. Klippel, Atugen, Berlin, Germany), a mAb specific for an N-terminal epitope in p85 $\alpha$  (clone AB6; Upstate Biotechnology, Charlottesville, VA), and a rabbit antiserum that recognizes all class I $\alpha$  PI3K regulatory isoforms (anti-pan-p85, 06-195; Upstate Biotechnology). To detect p85 $\beta$ , lysates were subjected to partial immunodepletion with mAb anti-p85 $\alpha$  (three successive immunoprecipitations) before blotting with anti-pan-p85. As loading controls, mAbs specific for  $\beta$ -actin or  $\alpha$ -tubulin (clones AC15 and DM1A; Sigma-Aldrich) were used. Band intensity was quantitated using NIH Image 1.61.

### Purification and culture of B and T cells

B and T cells were purified via negative selection on MACS columns (Miltenyi Biotec, Auburn, CA), as described (11), using anti-CD43 beads or the pan T cell isolation kit, respectively. Purity was verified to be comparable between wild-type and knockout cells, and above 90% via FACS analysis. For all assays, cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 5 mM HEPES, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and 50  $\mu$ M 2-ME. B cells were stimulated with anti-IgM (F(ab')<sub>2</sub>; Jackson ImmunoResearch Laboratories, West Grove, PA), anti-CD40 (clone HM40-3; BD Biosciences), LPS (serotype 0127:B8; Sigma-Aldrich), and IL-4 (R&D Systems). T cells were stimulated with anti-CD3 (clone 2C11; Southern Biotechnology Associates), anti-CD28 (clone 37.51; BD Biosciences), and IL-2 (R&D Systems). To inhibit PI3K pharmacologically, LY294002 (Calbiochem, San Diego, CA) was added at a final concentration of 5  $\mu$ M. For thymidine incorporation assays,  $5 \times 10^4$  T or B cells were stimulated in triplicate wells of 96-well plates (Falcon, Bedford, MA) with various amounts of the above listed stimuli, and they were allowed to proliferate for 48 h. At this point, 1  $\mu$ Ci of [<sup>3</sup>H]thymidine was added, and 16 h later it was harvested onto filter mats with a Tomtec (Hamden, CT) harvester. Filters were counted with a BetaPlate system (Wallac, Turku, Finland). To label cells with CFSE, splenocytes were first washed three times in HBSS. Next, CFSE was added at a concentration of 2.5  $\mu$ M to cells in HBSS for 8 min. The cells were then washed three times in the medium described above, counted, and resuspended at a concentration of  $1 \times 10^6$  cells/ml.

### Immunizations and ELISA

Mice were injected i.p. with either 100  $\mu$ l of nitrophenol (NP)-Ficoll or NP-OVA (Biosearch Technologies, Novato, CA), which was precipitated in 10% alum at a concentration of 1.5 mg/ml. Serum was obtained from tail bleeds of naive and injected mice. To measure levels of serum IgM, IgG1, or IgG2a, ELISA was conducted on duplicate samples of sera using plate-bound NP-BSA (Biosearch Technologies) as a capture reagent. Abs specific for NP were detected with anti-mouse IgM, IgG1, or IgG2a using HRP-conjugated Abs (Zymed Laboratories, South San Francisco, CA). Ab-

sorbance was read at 405 nm, and dilutions that yielded signals within a linear range were used for data analysis.

### Activation-induced cell death (AICD) assays

AICD was assessed by first stimulating splenocytes ( $1 \times 10^6$  cells) for 72 h with soluble anti-CD3 (2  $\mu$ g/ml) and IL-2 (50 U/ml) in 24-well plates in 1 ml. The cells were then spun in a Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) density gradient to eliminate dead cells. They were then washed in HBSS, counted, and restimulated in 96-well plates (Falcon) at a concentration of  $1 \times 10^6$  cells/ml with plate-bound anti-CD3 (12  $\mu$ g/ml), or incubated with IL-2 alone. After 6 h, the cells were stained with annexin V to measure apoptosis in CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

### Intracellular detection of phosphorylated S6

Purified T cells were stimulated with plate-bound anti-CD3 or anti-CD3 and anti-CD28, as described above. To detect phosphorylated S6, cells were fixed and permeabilized using the Cytofix/Cytoperm kit from BD Biosciences. A rabbit polyclonal Ab specific to Ser<sup>235/236</sup>-phosphorylated S6 (2211; Cell Signaling, Beverly, MA) was then added, followed by a FITC-conjugated goat anti-rabbit IgG Ab (F0382; Sigma-Aldrich). After washing, the cells were analyzed via FACS, as described above.

### Mouse hepatitis virus (MHV) infections

Mice were injected i.p. with  $2 \times 10^5$  PFU MHV (strain J2.2V-1) suspended in 500  $\mu$ l of sterile HBSS and sacrificed 5, 7, and 12 days postinfection. Splenocytes were isolated, as described above, and stained for flow cytometric analysis with the following reagents: anti-CD8 PerCP (BD Biosciences), anti-CD4 APC (Ebioscience), and PE-conjugated Db/S510-518 MHC class I tetramer, which is used for the identification of CD8 T cells specific for viral spike protein Ag (21).

### Microarray analysis

Splenocytes ( $2 \times 10^7$  in 20 ml) were activated with anti-CD3 at a concentration of 2  $\mu$ g/ml and 50 U/ml IL-2 for 60 h. T cells were then purified by a negative selection density gradient from SpinSep (StemCell Technologies, Vancouver, BC, Canada). This allowed us to maintain the cells at 4 degrees throughout purification and remove dead cells. Purity was verified to be greater than 95% via FACS. Resting T cells were purified from spleens in the same manner. T cells were lysed in TRIzol (Invitrogen, Carlsbad, CA), and lysates were pooled from three wild-type or three p85 $\beta$ -deficient mice. Total RNA was purified from the TRIzol with RNeasy (Qiagen, Valencia, CA), and 5  $\mu$ g was used for preparation of target for array hybridization. Affymetrix GeneChip murine genome U74Av2 (Affymetrix, Santa Clara, CA) was used. Target preparation and hybridization were conducted as described in the Affymetrix technical manual. Briefly, reverse transcription was performed on 5  $\mu$ g of total RNA using SuperScript II (Invitrogen) and an oligo(dT)<sub>24</sub> primer with a T7 RNA polymerase promoter (Genset, Paris, France). After second-strand synthesis, the double-stranded cDNA was purified by extraction with phenol-chloroform-isoamyl alcohol and recovered by ethanol precipitation. Bioarray HighYield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) was used for the production of biotin-labeled cRNA targets by in vitro transcription from T7 RNA polymerase promoters. In vitro transcription products were purified with an RNeasy to remove unincorporated NTPs and fragmented to various sizes (50–200 bases) by incubation at 94°C for 35 min. The fragmented sample cRNA was hybridized for 16 h at 45°C to an oligonucleotide array in the GeneChip Fluidics Station 400 (Affymetrix). The array underwent a series of nonstringent and stringent washes and was stained with streptavidin-conjugated PE. Probe arrays were scanned with an Agilent GeneArray Scanner (Agilent Technologies, Palo Alto, CA). The results were analyzed with Affymetrix Microarray Suite Version 5.0, and the expression levels of probe sets were computed. Three independent pools of RNA from three wild-type and p85 $\beta$ -deficient mice were included in the analysis. Data were analyzed further using D-CHIP (<http://www.dchip.org>), CyberT (<http://visitor.uci.edu/>), and GeneSpring (Silicon Genetics, Redwood City, CA) software, as described previously (22).

### Caspase-6 enzyme assays

Splenocytes were activated and T cells were purified in the same manner, as described in the microarray experiments. From here they were lysed and mixed with VEID-7-amino-4-trifluoromethyl-coumarin (50  $\mu$ M), a fluorogenic substrate of caspase-6 (buffer and substrate purchased from BioVision Research Products, Palo Alto, CA). Samples were analyzed on a fluorometer equipped with a 400-nm excitation filter and 505-nm emission

filter (Spex Fluorolog, Jobin Yvon, Middlesex, U.K.), and fluorescence values were normalized in terms of cell number.

### Statistical analysis

Statistical significance of microarray data was calculated using the Cyber *t* test, as previously described (22). For all other assays, a two-tailed paired Student's *t* test was conducted to determine statistical significance.

## Results

### Lymphocyte development

The generation and basic physiological description of p85 $\beta$ -deficient (Pik3r2 null) mice were described elsewhere (20). To determine whether the loss of p85 $\beta$  had any effect on lymphopoiesis, we analyzed primary and secondary lymphoid organs of wild-type and knockout mice. As shown in Table I, B cells developed normally in the absence of p85 $\beta$ . The percentages of cells expressing IgM, IgD, B220, and CD5 in spleen and peritoneal cavity were indistinguishable in wild-type and p85 $\beta$ -deficient littermates. Additionally, T cell development was grossly normal in the absence of p85 $\beta$ , as assessed by comparing ratios of CD4/CD8 single-positive/double-positive/double-negative cells in the thymus. The numbers and ratios of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the secondary lymphoid organs were also indistinguishable in wild-type and p85 $\beta$ -deficient mice. Markers of activation, memory, and regulatory phenotype (CD25, CD69, CD44, and CD62L) were also found on similar percentages of both CD4 and CD8 splenic T cells in p85 $\beta$ -deficient mice relative to wild type. These data are enumerated in Table II.

### Class I<sub>A</sub> PI3K subunit expression

Disruption of class I<sub>A</sub> PI3K genes is often associated with compensatory changes in expression of other isoforms (11, 23–25). However, analysis of thymocyte lysates showed that expression of p85 $\alpha$  and its splice variants, as well as the catalytic subunits p110 $\alpha$ , p110 $\beta$ , and p110 $\delta$  was unaltered in the absence of p85 $\beta$  (Fig. 1). The absence of p85 $\beta$  protein was confirmed by immunoblotting (Fig. 1).

### B cell function

The defects in B cell development and function in p85 $\alpha$ -deficient mice (11, 17) highlighted the importance of PI3K signaling in B cells, but did not distinguish whether other PI3K regulatory isoforms also have unique required roles. To investigate the effects of p85 $\beta$  deletion on B cell function, we measured proliferation in vitro and Ab production in vivo. As seen in Fig. 2A, p85 $\beta$ -deficient

B cells proliferated to a similar extent as wild type when stimulated with different doses of anti-IgM, in the absence or presence of IL-4. Treatment with anti-CD40, anti-CD40 and IL-4, or LPS also induced comparable levels of proliferation in wild-type and p85 $\beta$ -deficient B cells (Fig. 2A, lower panel). Each of these responses can be blocked by global PI3K inhibitors or loss of p85 $\alpha$  (Fig. 2A) (11).

Fig. 2B shows the primary and secondary Ab responses to both T-dependent and T-independent Ags. After a single injection with the T-dependent Ag, NP-OVA, there was a robust response of hapten (NP)-specific IgM in both wild-type and p85 $\beta$ -deficient mice. Furthermore, serum levels of IgM, IgG1, and IgG2a were nearly identical in wild-type and p85 $\beta$ -deficient mice in secondary responses. IgM production was also comparable when mice were immunized with NP-Ficoll, a T-independent type II Ag (Fig. 2B). These results led us to conclude that p85 $\beta$  is dispensable for B cell function in vitro and in vivo.

### T cell function

T cell proliferation was measured initially in splenocytes labeled with the cell division tracker dye CFSE. Seventy-two hours after stimulation with soluble anti-CD3 and IL-2, cells were analyzed by FACS for the number of cell divisions and the percentage of dying cells (annexinV positive) in the CD4 and CD8 subsets. Fig. 3A shows a histogram of CFSE intensity from a representative experiment, with quantitation of the percentage of cells that are in each division state. Both CD4 and CD8 T cells from knockout mice showed enhanced percentages of cells that had divided from four to seven times, and reduced percentages that had divided one to three times. To normalize for experimental variability for individual peaks, the percentages of viable (annexin V<sup>−</sup>) cells that had divided more than three times were grouped and averaged over four experiments, and the data are depicted graphically in Fig. 3B. These results demonstrated a significant increase in the percentage of viable p85 $\beta$ -deficient T cells that had undergone four or more rounds of replication, a difference that was evident in both CD4<sup>+</sup> and CD8<sup>+</sup> subsets. However, these differences were less pronounced when lower doses of anti-CD3 were used, or if no exogenous IL-2 was added (data not shown). In some, but not all experiments with anti-CD3 plus IL-2, a decrease in the percentage of annexin-V<sup>+</sup> cells was observed in the p85 $\beta$ -deficient samples (data not shown); it is possible that the failure to observe uniform

Table I. Lymphocyte development in the absence of p85 $\beta$ <sup>a</sup>

Organ	Cell Type	Wild Type %	Knockout %
Spleen	B220 <sup>+</sup>	57.7 ± 5.5	58.6 ± 1.3
	IgM <sup>low</sup> /IgD <sup>high</sup>	36.0 ± 2.3	31.3 ± 0.9
	Thy-1.2 <sup>+</sup>	30.4 ± 2.3	28.6 ± 2.2
Lymph node	B220 <sup>+</sup>	27.5 ± 1.3	31.9 ± 1.3
	Thy-1.2 <sup>+</sup>	64.1 ± 1.6	60.0 ± 1.2
	CD4 <sup>+</sup>	49.8 ± 2.0	48.9 ± 1.3
	CD8 <sup>+</sup>	17.0 ± 1.1	15.0 ± 0.18
Peritoneum <sup>c</sup>	CD5 <sup>+</sup> /B220 <sup>+</sup> (all cells)	32.6 ± 1.5	30.2 ± 8.8
	CD5 <sup>+</sup> /B220 <sup>+</sup> (lymphoid cells)	56.9 ± 7.1	60.6 ± 1.4
Thymus	CD4 <sup>+</sup> /CD8 <sup>−</sup>	4.9 ± 0.9	4.9 ± 0.7
	CD4 <sup>+</sup> /CD8 <sup>+</sup>	82.8 ± 1.4	83.1 ± 2.3
	CD4 <sup>+</sup>	9.1 ± 2.5	7.0 ± 2.0
	CD8 <sup>+</sup>	3.1 ± 0.3	3.1 ± 0.5

<sup>a</sup> Results are the average of three to five mice, expressed as an average percentage ± SEM.

<sup>b</sup> IgM<sup>low</sup>/IgD<sup>high</sup> cells represent mature recirculating B cells.

<sup>c</sup> "All cells" and "lymphoid cells" refer to the gating used in FACS analysis, as judged by forward and side scatter.



Table II. Activation/Memory markers in p85β<sup>-/-</sup> T cells<sup>a</sup>

Genotype	Wild Type		Knockout	
	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>
Cell Type				
CD44 <sup>+</sup> /CD62L <sup>high</sup>	19.1 ± 2.7	9.1 ± 2.0	20.8 ± 2.0	9.5 ± 1.0
CD44 <sup>+</sup> /CD62L <sup>low</sup>	16.8 ± 1.0	16.1 ± 2.6	16.4 ± 1.4	15.8 ± 2.0
CD44 <sup>-</sup> /CD62L <sup>high</sup>	55.5 ± 3.0	54.0 ± 5.4	55.1 ± 5.1	57.9 ± 6.5
CD44 <sup>-</sup> /CD62L <sup>low</sup>	8.6 ± 2.2	20.8 ± 6.7	7.8 ± 2.5	16.9 ± 5.1
CD25 <sup>+</sup> /CD69 <sup>+</sup>	0.7 ± 0.2	1.2 ± 0.5	0.7 ± 0.2	1.3 ± 0.7
CD25 <sup>+</sup> /CD69 <sup>-</sup>	5.9 ± 1.3	0.7 ± 0.2	5.6 ± 1.2	0.6 ± 0.2

<sup>a</sup> Results are the average of four pairs of mice, expressed as an average percentage ± SEM.

decreases in cell death was due to variability in phagocytic scavenging of apoptotic cells by macrophages in these splenocyte cultures.

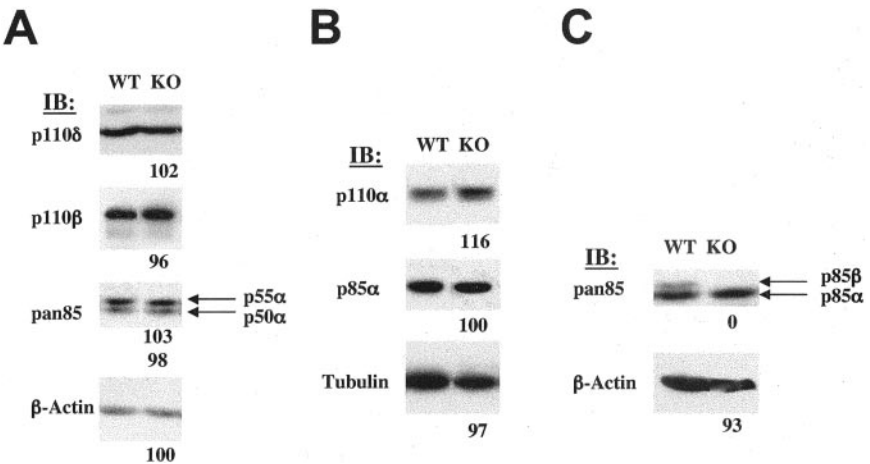
Increased T cell proliferation in splenocyte cultures could be the result of altered function of accessory cells rather than a cell-autonomous effect in T cells. However, we also observed increased proliferation when purified T cells were stimulated with plate-bound anti-CD3 in the absence or presence of IL-2. As shown in Fig. 4A, there was a statistically significant enhancement ( $p < 0.05$ ) in thymidine incorporation in the p85β-deficient T cells at every dose of stimulation, except the lowest of anti-CD3 alone (1.3 μg/ml). The extent of the differences was most marked at higher doses of stimulation. At the highest dose of anti-CD3, p85β-deficient T cells incorporated more than twice as much thymidine as the T cells from wild-type littermates.

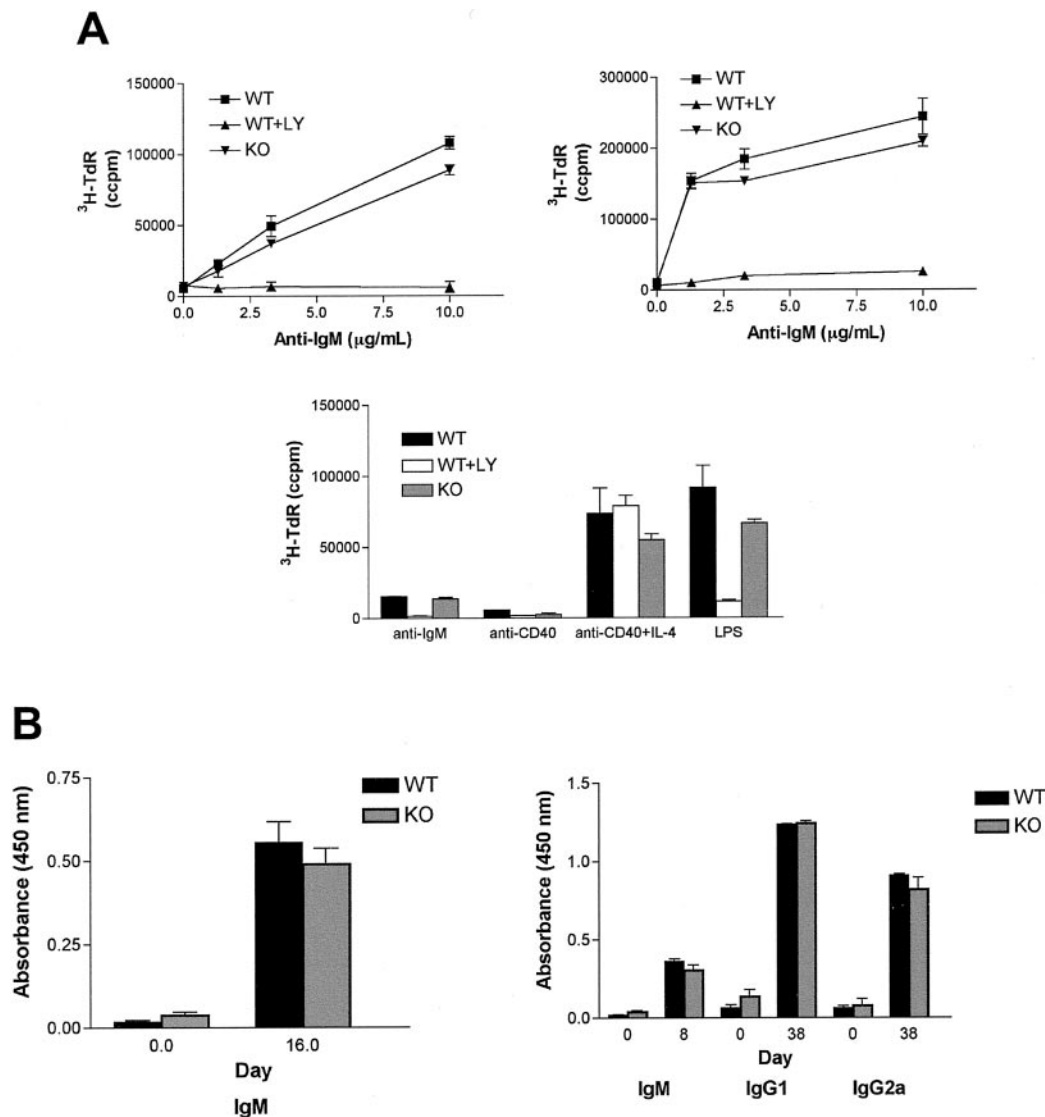
We also measured the response of purified T cells to a range of concentrations of exogenous IL-2, in the absence or presence of anti-CD3 (12 μg/ml). IL-2 alone was insufficient to produce robust levels of thymidine incorporation in either wild-type or p85β-deficient T cells (Fig. 4A), consistent with the low percentage of naive cells expressing the high affinity IL-2R CD25 (Table II). However, the weak response to high dose IL-2 (50 or 100 U/ml) was consistently higher in the p85β-deficient T cells. The combination of IL-2 and anti-CD3 boosted proliferation above anti-CD3 alone within the range of 20–100 U/ml IL-2, and this increase occurred to a greater extent in the p85β<sup>-/-</sup> T cells (Fig. 4A). Together, these data suggest that p85β-deficient T cells are more responsive to IL-2. In contrast, the response to anti-CD3 plus anti-CD28 in p85β-deficient T cells was not appreciably different from wild type (Fig. 4B). Additionally, p85β-deficient T cells were shown to be PI3K dependent, as treatment with LY 294002 blocked proliferation to a similar extent as wild-type T cells (Fig. 4C).

To determine whether enhanced thymidine incorporation was due to an increase in the fraction of cycling cells or a decrease in cell death, we used propidium iodide staining to analyze DNA content of stimulated T cells. Under conditions (12 μg/ml anti-CD3 + 50 U/ml IL-2) that gave the largest increase in thymidine incorporation in p85β-deficient T cells, there was no statistically significant difference in percentages of cells in G<sub>1</sub>, S, or G<sub>2</sub> phases of cell cycle (Fig. 4D). However, there was a marked decrease in the percentage of apoptotic cells 48 h after stimulation, as determined by subdiploid DNA content. This time point is coincident with the point in which [<sup>3</sup>H]thymidine was added to cells in Fig. 4A. These results indicate that elevated proliferation in p85β-deficient T cells is the result of an increased number of live, dividing cells rather than a greater fraction of cells in S phase.

To determine whether differences in T cell activation in vitro were relevant to T cell function in vivo, we studied the immune response to a virus. The numbers and phenotypes of lymphocyte populations in the spleen were monitored at different times following i.p. infection with MHV. The immune response to this virus is well characterized, and virus-specific CD8<sup>+</sup> T cells can be detected by MHC tetramer reagents. At 5 and 7 days postinfection, spleens were large in both wild-type and p85β-deficient mice. There was no difference in total splenocytes or percentages of different T cell subsets (data not shown), suggesting that the initial phases of adaptive viral immunity were intact. However, at 12 days postinfection, the p85β-deficient mice showed sustained elevation in splenic lymphocytes, whereas splenomegaly observed in wild-type mice at earlier time points had resolved (Table III). We observed a greater than 2-fold increase in the number of total splenocytes, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and tetramer-positive CD8<sup>+</sup> T cells. These findings establish a physiological role for p85β in limiting T cell expansion in vivo following viral infection.

**FIGURE 1.** Normal expression of class I<sub>A</sub> PI3K isoforms in the absence of p85β. Thymocyte lysates from wild-type and knockout mice were probed with Abs specific for the catalytic and regulatory subunits of PI3K. A–C, Represent different sets of lysates. To detect p85β, lysates were partially depleted of endogenous p85α, because the greater sensitivity of this Ab to p85α (C.M.Y., unpublished observations) normally obscures the p85β band. Equal levels of protein loading were confirmed by probing for β-actin or tubulin, as shown at the bottom. Numbers below the knockout lanes represent the band intensity relative to the wild-type sample (equal = 100). Data are representative of three littermate pairs.





**FIGURE 2.** Normal B cell proliferation and Ab secretion in  $p85\beta^{-/-}$  mice. *A*, B cells were stimulated with various concentrations of the  $F(ab')_2$  of  $\alpha$ IgM with or without IL-4 (*right* and *left* panels, respectively). Proliferation was measured by [ $^3$ H]thymidine incorporation, and a representative of five experiments is shown. As a comparison for the effects of global PI3K inhibition, wild-type cells were treated at all points with LY294002. The *lower* panel compares proliferation with anti-IgM alone (10  $\mu$ g/ml) with that of anti-CD40 (1  $\mu$ g/ml), LPS (10  $\mu$ g/ml), and anti-CD40 (1  $\mu$ g/ml) + IL-4. The apparent decrease in anti-CD40 response in this experiment was not observed in other experiments. *B*, Wild-type and knockout mice were challenged with Ags via i.p. injection of the Ags NP-Ficoll (*left* panel) or NP-OVA (*right* panel). NP-specific Abs were detected by ELISA and levels of IgM, IgG1, or IgG2a compared with that present in serum from naive mice (prebleeds). Data are shown as the mean  $\pm$  SEM of four littermate pairs.

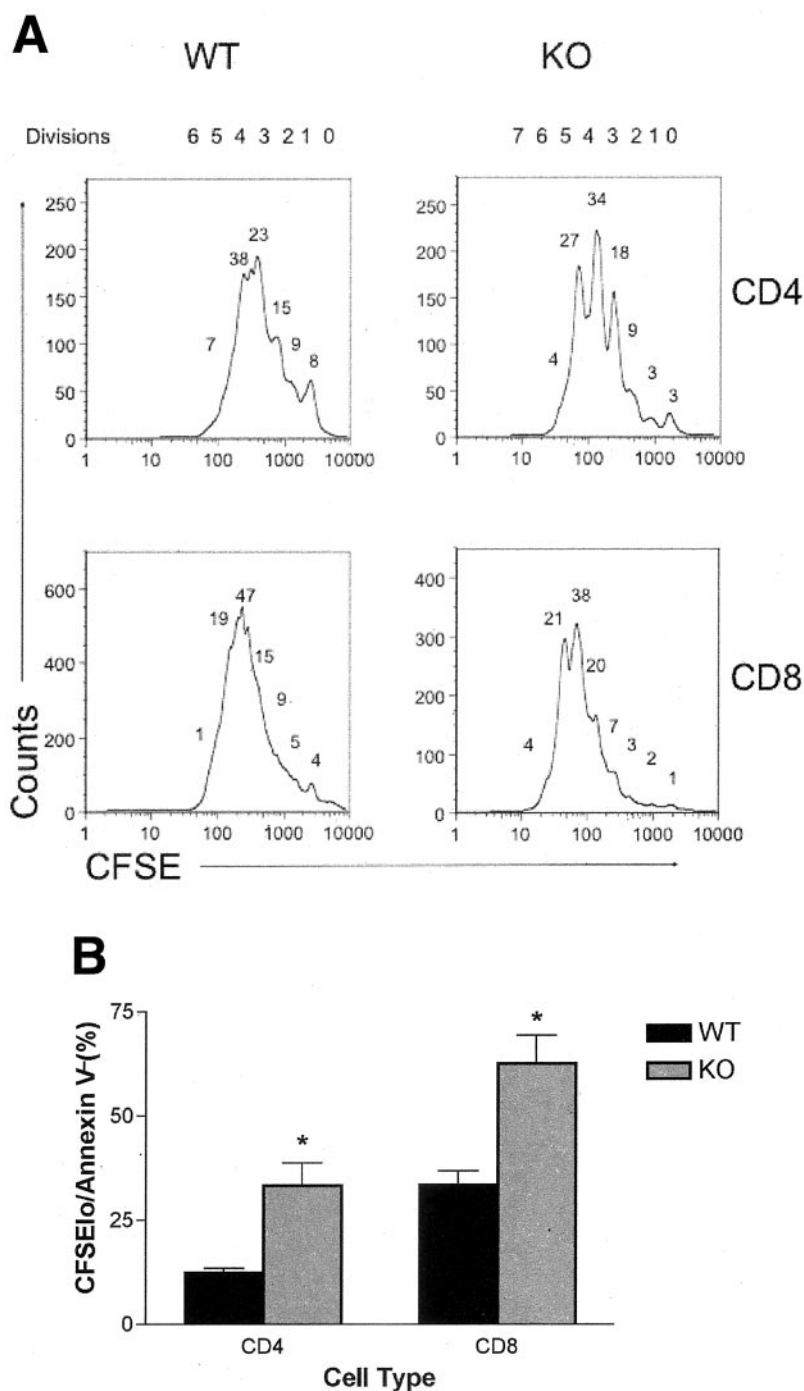
Sustained or repeated T cell stimulation leads to AICD via a Fas-dependent process (26). AICD is promoted by IL-2-dependent signals and is important for maintaining T cell homeostasis and preventing autoimmunity (27). To test whether increased expansion of  $p85\beta$ -deficient T cells *in vitro* was the result of impaired AICD, we used a standard assay in which activated T cells were either rested or restimulated with plate-bound anti-CD3 (28). In this system, we observed comparable increases in death of  $p85\beta$ -deficient and wild-type T cells (Fig. 4E). Additionally, no differences in apoptosis were observed when activated T cells were treated with membrane-bound Fas ligand, nor in passive death in unstimulated splenocyte cultures (data not shown). Thus, the Fas-dependent process of AICD could not account for the decreased death in  $p85\beta$ -deficient T cells. This finding is consistent with a previous study uncoupling IL-2-dependent PI3K activation from sensitization to AICD (27).

Differences in proliferation might be attributed to direct alterations in the amounts of PI3K products in T cells lacking  $p85\beta$ .

Because direct measurement of 3-phosphoinositides is technically formidable, phosphorylation of proteins downstream of PI3K is often used as a surrogate readout of PI3K signaling output. Phosphorylation of the S6 ribosomal subunit is dependent on PI3K catalytic function (29) and can be measured in a FACS-based assay (Fig. 5) (29, 30). When T cells were stimulated with anti-CD3 or anti-CD3 and anti-CD28, the percentage positive for phospho-S6 and the mean fluorescence index of the positive cells were nearly identical in wild-type and  $p85\beta$ -deficient T cells across multiple time points (Fig. 5). S6 phosphorylation was blocked by treatment with LY294002 (Fig. 5).

#### Caspase-6 up-regulation

Screening of candidate survival regulatory genes in activated T cells, including Fas ligand and Bcl- $x_L$ , did not reveal differences in protein expression (data not shown). To gain an unbiased, global view of the expression of proliferation and survival genes, we used DNA microarrays. Viable T cells were purified from splenocyte



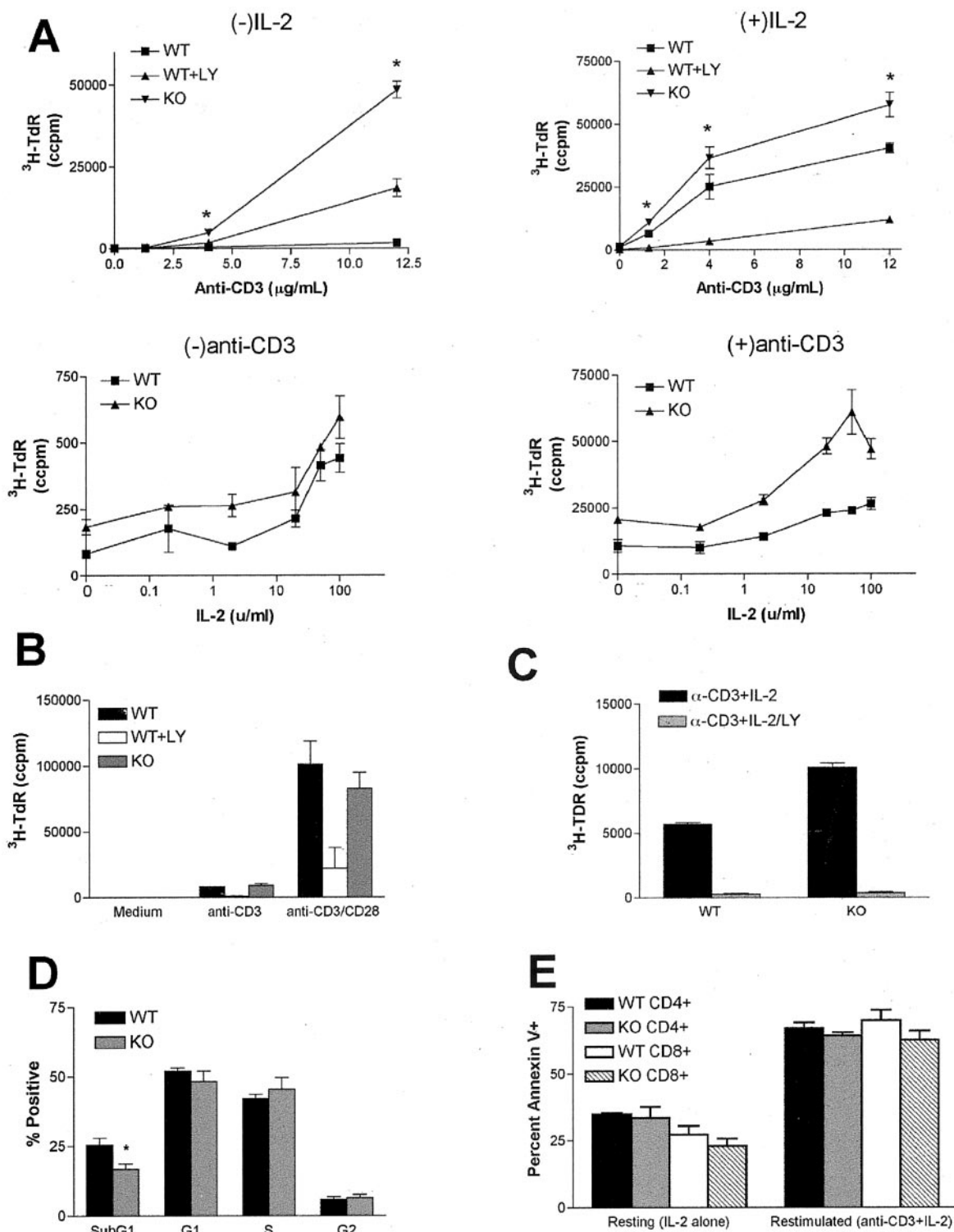
**FIGURE 3.** Enhanced cell division in p85 $\beta$ -deficient T cells. The mitotic history of splenic T cells stimulated with anti-CD3 (2  $\mu$ g/ml) and IL-2 (50 U/ml) was measured with the cell division tracker dye CFSE. **A**, At 72 h, histograms of CFSE intensity in T cells were plotted based on gating for CD4 and CD8 subsets in wild-type (WT) and knockout (KO) mice. FlowJo software was used to quantitate the percentage of cells in each peak (numbers shown above). The number of divisions represented by each peak is shown above the histograms. A representative experiment of four is shown. It should be noted that the apparent decrease in the event rate for CD8 cells in the KO is not representative of all four experiments. **B**, CFSE data are shown in graphical format as the average percentage of viable (annexin V<sup>-</sup>) CFSE<sup>low</sup> CD4 and CD8 T cells ( $\pm$ SEM) of four independent experiments, in which CFSE<sup>low</sup> represents cells that have divided more than three times. \*,  $p < 0.05$ .

cultures 60 h following stimulation with anti-CD3 plus IL-2, and RNA was isolated. The complete microarray dataset will be available on our lab web site (<http://mbb.bio.uci.edu/fruman/>); in this study, we report that caspase-6 showed the most statistically robust difference in mRNA expression between stimulated wild-type and p85 $\beta$ -deficient T cells ( $p < 0.001$ ). The product of the caspase-6 gene has been described as executioner caspase during apoptosis, but it has been shown in some systems that it can also function to activate molecules upstream or parallel in the caspase-dependent death pathway (31, 32). Additionally, increased caspase-6 expression can lower the apoptotic threshold in response to some death signals (33). Fig. 6A depicts fluorescence values from the microarray experiments after application of the D-CHIP modeling algorithm (34). Consistent with previous studies of human T cells (35),

murine caspase-6 was up-regulated upon stimulation of wild-type cells. Strikingly, the elevation of caspase-6 mRNA was much attenuated in activated p85 $\beta$ -deficient T cells. To establish that caspase-6 function was also impacted at the protein level, we used a specific fluorogenic substrate to measure caspase-6 enzyme activity in lysates of activated T cells. This experiment showed that the amount of caspase-6 activity was significantly decreased in stimulated p85 $\beta$ -deficient T cells, as compared with wild-type controls (Fig. 6B).

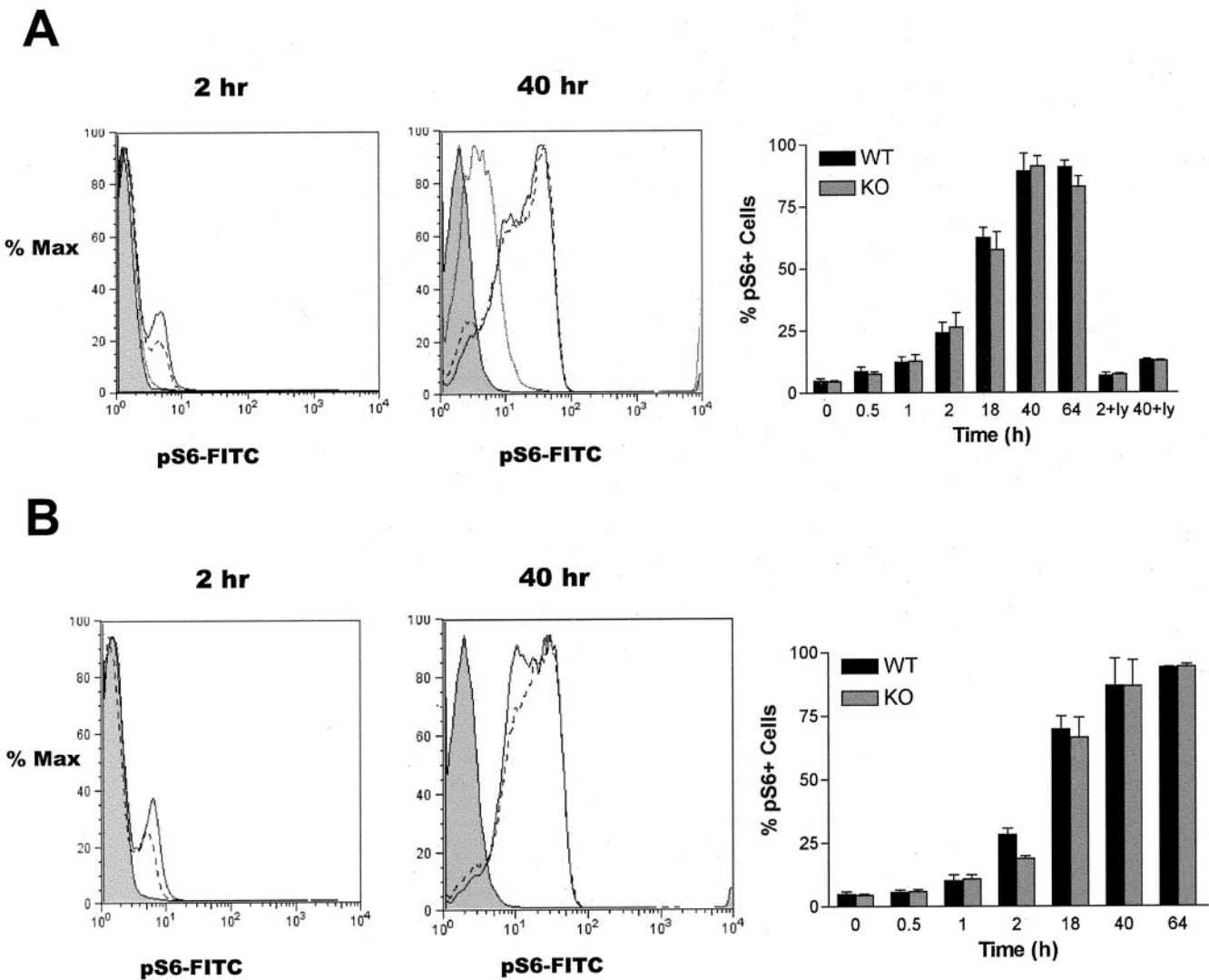
## Discussion

PI3K has been shown to be important for survival, activation, and proliferation in a variety of cell types. Ag receptor stimulation of both T and B cells leads to PIP<sub>3</sub> accumulation (5, 6, 36), and



**FIGURE 4.** Increased thymidine incorporation in p85 $\beta$ -deficient purified T cells correlates with decreased death. *A, Top*, Purified T cells were stimulated with various amounts of plate-bound  $\alpha$ CD3 with or without IL-2 (*right* and *left* panels, respectively). Wild-type (WT) cells treated with LY294002 were used to demonstrate the effects of global PI3K inhibition. *Bottom*, T cells were stimulated with a titration of IL-2 doses in the absence or presence of  $\alpha$ CD3 (*left* and *right* panels, 12  $\mu\text{g/mL}$ ). Representatives from five experiments are shown. Statistical significance was calculated by normalizing the cpm of five to seven experiments. *B*, T cells were stimulated with anti-CD3 (4  $\mu\text{g/mL}$ ) or both anti-CD3 (4  $\mu\text{g/mL}$ ) and anti-CD28 (10  $\mu\text{g/mL}$ ). A representative of four experiments is shown. *C*, T cells from wild-type and knockout (KO) mice were stimulated, as in *A*, with anti-CD3 (12  $\mu\text{g/mL}$ ) and IL-2 (50 U/ml) with or without LY (10  $\mu\text{M}$ ). Data are representative of four independent experiments. *D*, T cells were stimulated, as in *C*. At 48 h, the cells were fixed and stained with propidium iodide to assess DNA content. Data are shown as the mean percentage of cells found in G<sub>1</sub>, S, G<sub>2</sub>, or subdiploid phases,  $\pm$ SEM ( $n = 4$ ). G<sub>1</sub>, S, and G<sub>2</sub> phases are expressed as a percentage of live cells. *E*, AICD was assessed as follows: after an initial 72-h stimulation of splenocytes (as in Fig. 3), dead cells were removed. Cells were either cultured in IL-2 alone for 6 h (designated resting), or restimulated with plate-bound  $\alpha$ CD3 (12  $\mu\text{g/mL}$ ) for the same amount of time. Apoptosis in either condition was measured in both CD4 and CD8 T cells by flow cytometry, as indicated by annexin V binding. \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ .





**FIGURE 5.** Normal biochemical activation of the PI3K downstream target S6 in p85β-deficient T cells. T cells were stimulated with anti-CD3 alone (12 μg/ml, A) or anti-CD3 and anti-CD28 (12 and 10 μg/ml, B) for various time points. Cells were fixed, permeabilized, and stained for phosphorylated S6. Representative experiments showing FACS histograms for unstimulated (filled graph), wild-type (WT) (solid lines), WT + LY (thin lines), and knockout (dashed lines) at 2 h (left histograms) or 40 h (right histograms). Bar graphs enumerate the means ± SEM of three pairs of mice for the percentage of T cells positive for phosphorylated S6 at all time-observed points.

receptors for cytokines and costimulatory molecules further boost PI3K activation (37, 38). Early attempts to define the role of PI3K signaling in T cells yielded conflicting conclusions, in part due to the use of Jurkat cells and other transformed cell lines (39). It has since been shown that Jurkat cells maintain constitutively high levels of PIP<sub>3</sub> due to the absence of lipid phosphatases (39). Studies of primary T cells using PI3K inhibitors (wortmannin, LY294002) have generally supported a role for PI3K activity in proliferation and survival (7, 11, 12). A major limitation to these experiments is that wortmannin and LY294002 globally inhibit nearly all PI3K catalytic isoforms. So, while it was clear that PI3K activity was essential for T cell survival, these studies could not distinguish the roles of individual catalytic and regulatory isoforms.

An important advance was the generation of a mouse strain in which a catalytically inactive form of p110δ was knocked into the wild-type allele (14). In these mice, T cell activation was significantly reduced, demonstrating that class I<sub>A</sub> PI3K signaling is essential for T cell activation. T cell defects were not reported by two separate groups that generated p110δ null alleles (18, 19); how-

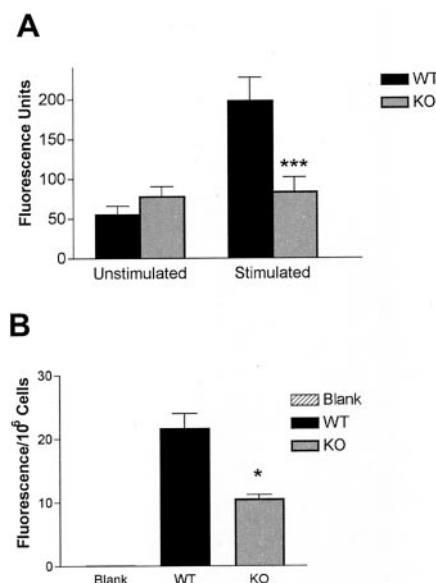
ever, in contrast to mice with the kinase-dead knockin mutation, compensatory up-regulation of other class I<sub>A</sub> PI3K catalytic isoforms may have occurred in p110δ null T cells. We have not observed reduced proliferation in T cells lacking either p85α or p85β. Together, these findings suggest that activation of class I<sub>A</sub> PI3K in T cells can be mediated by either p85α or p85β. In this respect, there is redundancy in the function of the two regulatory subunit genes.

Nevertheless, changes in lymphocyte function are observed in mice lacking either p85α or p85β, demonstrating unique roles for

Table III. Response to MHV infections in wild-type (WT) and knockout (KO) mice 12 days postinfection<sup>a</sup>

Genotype	Splenocytes	CD4 <sup>+</sup> Cells	CD8 <sup>+</sup> Cells	Tetramer <sup>+</sup> Cells
WT	70 × 10 <sup>6</sup>	10.8 × 10 <sup>6</sup>	14 × 10 <sup>6</sup>	1.3 × 10 <sup>6</sup>
KO	180 × 10 <sup>6</sup>	20 × 10 <sup>6</sup>	39.1 × 10 <sup>6</sup>	3.2 × 10 <sup>6</sup>

<sup>a</sup> Results are the average of four mice per group. Similar results were obtained in a repeat experiment.



**FIGURE 6.** Impaired transcriptional up-regulation and enzymatic activity of caspase-6 in p85 $\beta$ -deficient T cells. *A*, Microarray fluorescence values are shown from T cells that were unstimulated or stimulated for 60 h, as in Fig. 3. Results are the mean D-CHIP-filtered fluorescence values from three independent pools of mRNA from wild-type or knockout mice. *B*, T cells were isolated from stimulated splenocytes, as in *A*. Protein lysates were made and subjected to caspase-6 enzyme assays using a fluorogenic substrate, with lysis buffer as the blank. Data are shown as the average fluorescence per 10<sup>6</sup> cells,  $\pm$  SEM ( $n = 3$ ). \*,  $p < 0.05$ ; \*\*\*,  $p < 1 \times 10^{-5}$ .

individual class I $\alpha$  PI3K regulatory isoforms in the two cell types. Whereas mice lacking p85 $\alpha$  show severe defects in B cell development and proliferation, the T cells from these mice are relatively normal (11, 17). In contrast, we have shown in this work that mice that lack p85 $\beta$  exhibit normal B cell function, but enhanced T cell expansion in vitro and in vivo. Of note, no overt signs of autoimmunity were observed when three wild-type and three p85 $\beta$ -deficient mice were sacrificed and analyzed at 14 mo of age (data not shown). The absence of spontaneous autoimmunity is consistent with the normal Fas-dependent AICD observed in p85 $\beta$ -deficient T cells. However, further work is necessary to determine whether loss of p85 $\beta$  alters susceptibility in established models of autoimmunity.

In a great variety of cellular systems, reducing PI3K function diminishes proliferation and/or promotes cell death (2–4). Consequently, it was surprising that T cells lacking p85 $\beta$  showed an increase in proliferation and a decrease in cell death. One possibility is that PI3K signaling via p85 $\beta$  triggers a feedback mechanism that limits T cell expansion by sensitizing cells to apoptosis. It is becoming evident that there are multiple death pathways in activated T cells (26, 40). However, p85 $\beta$  does not appear to be essential for two major pathways that have been termed AICD and activated T cell-autonomous death. We have shown that the Fas-dependent process of AICD is unaltered in p85 $\beta$ -deficient T cells. Decreased activated T cell-autonomous death is associated with depletion of growth factors and would not appear to apply in these studies, in which the greatest increases in proliferation of p85 $\beta$ -deficient T cells were observed when maximal concentrations of anti-CD3 were applied in the presence of exogenous IL-2.

An initial clue to the mechanism of enhanced survival in p85 $\beta$ -deficient T cells might be our identification of a discrete member of apoptotic machinery that is differentially expressed (caspase-6). The level of caspase-6 expression, an executioner caspase, could in-

fluence the threshold of death signaling required to trigger apoptosis (33). We have not yet established the molecular link between p85 $\beta$  and caspase-6 up-regulation. Based on the greater divergence of the SH3, proline-rich, and RhoGAP homology domains of p85 $\alpha$  vs p85 $\beta$  relative to the highly conserved SH2 domains, it seems likely that distinct molecular interactions involving these domains are responsible for the unique functions of p85 $\beta$  in T cells. Indeed, it has been demonstrated that the regulatory subunits are differentially serine phosphorylated following TCR engagement (41), and that p85 subunits can serve as adapters to regulate cytokine transcription independently of p110 association (42). Given the apparently normal PI3K catalytic function, as judged by phosphorylation of the downstream protein S6 (Fig. 5), it is reasonable to propose that the loss of adapter functions unique to p85 $\beta$  might underlie the phenotype described in this work. Further structure-function analysis, combined with proteomics and transcriptional profiling are required to further define the mechanistic differences in function of class I $\alpha$  PI3K regulatory subunits in T cells.

Ag receptor-stimulated Ca<sup>2+</sup> mobilization is critical for proliferation in both T and B cells. Interestingly, the contributions of PI3K and other proteins to this process differ significantly in the two cell types. Genetic and biochemical studies of BCR signaling have led to the definition of a Ca<sup>2+</sup> signalosome (43, 44), a set of signaling components that come together at the membrane to promote the tyrosine phosphorylation and activation of phospholipase C (PLC), leading to hydrolysis of PIP<sub>2</sub> to generate the critical second messengers inositol 1,4,5-triphosphate and 1,2-diacylglycerol. Components of the B cell signalosome include the B cell linker protein (45–48), the p85 $\alpha$  regulatory and p110 $\delta$  catalytic isoforms of PI3K (11, 14, 17–19), the tyrosine kinase Btk (49–51) (although the related Tec isoform contributes in mice (52)), PLC- $\gamma$ 2 (44, 53, 54), and possibly the newly defined adapter protein linker of activated B cells (55). T cells seem to use a set of related, but distinct gene products to link the TCR to Ca<sup>2+</sup> mobilization (56). Thus, the role of Btk is served by the Tec family kinases Itk and Rlk; B cell linker protein and linker of activated B cells are replaced by SH2-containing leukocyte protein-76 and linker for activation of T cells; and PLC- $\gamma$ 1 rather than PLC- $\gamma$ 2 appears to be the primary isoform responsible for PIP<sub>2</sub> hydrolysis. Importantly, although PI3K inhibitors block T cell proliferation as they do in B cells, loss of p85 $\alpha$  has no apparent effect (11).

These observations led us to wonder whether the p85 $\beta$  isoform of PI3K might function in T cells in the same way that p85 $\alpha$  functions in the B cell signalosome. However, unlike the similar phenotypes of B cells lacking Btk or p85 $\alpha$ , T cells lacking Itk or p85 $\beta$  have very different characteristics. Itk-deficient T cells show impaired proliferation in response to anti-CD3 (57), whereas we show in this work that p85 $\beta$ -deficient cells have no defect in cell cycle progression, actually completing more rounds of division while exhibiting less death. Itk-deficient T cells have a defect in sustained Ca<sup>2+</sup> mobilization (58). In contrast, we have observed no impairment in Ca<sup>2+</sup> mobilization in T cells lacking p85 $\beta$  (data not shown). Indeed, we find that treatment with PI3K inhibitors has little effect on Ca<sup>2+</sup> mobilization in T cells stimulated by CD3 cross-linking (data not shown), as recently reported in an Ag-specific system (8), but contrasting with other reports using anti-CD3 (14). Both Itk- and p85 $\beta$ -deficient T cells can be activated by the combination of TCR and CD28 engagement (59). However, activated Itk-deficient cells show two additional defects: resistance to AICD, and a block in Th2 differentiation (60, 61). We have shown in this work that although p85 $\beta$ -deficient cells have a survival advantage, this does not appear to be the result of impaired AICD. Furthermore, unlike the Itk-deficient T cells, we have found no significant differences in the pattern of cytokines produced by purified CD4<sup>+</sup> T

cells or bulk spleen cell cultures stimulated with anti-CD3 (data not shown). Together, the available data underscore the differences between BCR and TCR signalosomes. They also suggest that Itk has a PI3K-independent contribution to Ca<sup>2+</sup> mobilization and proliferation following TCR ligation. Finally, the observation that PI3K inhibitors do not inhibit early activation markers, yet block cell division in T cells when added 9 h after Ag exposure (7), raises the possibility that PI3K is more important in T cells for cytokine-driven cell cycle progression than for early stages of activation.

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