The p85α Regulatory Subunit of Class IA Phosphoinositide 3-Kinase Regulates β-Selection in Thymocyte Development[1]

Fumiko Shiroki,* Satoshi Matsuda,*† Tomomitsu Doi,*† Mari Fujiwara,*† Yoshito Mochizuki,* Takashi Kadowaki,†‡ Harumi Suzuki,*§ and Shigeo Koyasu[2]*†

We examined the role of class IA PI3K in pre-TCR controlled β-selection and TCR-controlled positive/negative selection in thymic development. Using mice deficient for p85α, a major regulatory subunit of the class IA PI3K family, the role of class IA PI3K in β-selection was examined by injection of anti-CD3ε mAb into p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice, which mimics pre-TCR signals. Transition of CD4$^-$CD8$^-$ double-negative (DN) to CD4$^+$CD8$^+$ double-positive (DP) thymocytes triggered by anti-CD3ε mAb was significantly impaired in p85$\alpha^{-/-}$Rag-2$^{-/-}$ compared with p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice. Furthermore, DP cell numbers were lower in p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ TCR-transgenic mice than in DO11.10/Rag-2$^{-/-}$ mice. In addition, inhibition by IC87114 of the major class IA PI3K catalytic subunit expressed in lymphocytes, p110δ, blocked transition of DN to DP cells in embryonic day 14.5 fetal thymic organ culture without affecting cell viability. In the absence of phosphatase and tensin homolog deleted on chromosome 10, where class IA PI3K signals would be amplified, the DN to DP transition was accelerated. In contrast, neither positive nor negative selection in Rag-2$^{-/-}$TCR-transgenic mice was perturbed by the lack of p85α. These findings establish an important function of class IA PI3K in the pre-TCR-controlled developmental transition of DN to DP thymocytes. ***The Journal of Immunology,*** **2007, 178: 1349–1356.**

T cell development in the thymus is a highly controlled process beginning with the most immature thymocyte, termed CD4$^-$CD8$^-$ double-negative (DN)[3] (1–3). The DN progenitors can be subdivided into four different stages based on their CD44 and CD25 expression patterns. The earliest progenitors are CD44$^+$CD25$^-$ (DN1) cells, followed by CD44$^+$CD25$^+$, CD44$^-$CD25$^+$, and CD44$^-$CD25$^-$ (DN2–4, respectively) cells. After successful rearrangement of the TCRβ, the CD44$^-$CD25$^+$ (DN3) cells express a pre-TCR on their surface made up of the TCRβ protein and a pTα. After passing the first T cell developmental checkpoint, namely β-selection, DN3 cells make the transition to the CD4$^+$CD8$^+$ double-positive (DP) stage. This transition includes cell proliferation triggered by signals through the pre-TCR. The pre-TCR also triggers rearrangement of the *TCR*α gene, leading to the expression of TCR$\alpha\beta$ heterodimers on DP cells. These cells then go through a second checkpoint, termed positive and negative selection. DP cells receiving weak signals through the TCR survive and differentiate into mature CD4$^+$ or CD8$^+$ single-positive (SP) cells (positive selection), whereas those receiving strong signals are eliminated (negative selection). Furthermore, those incapable of recognizing appropriate MHC molecules fail to receive the TCR signal and are also eliminated (death by neglect) (1–3).

PI3Ks are lipid kinases that specifically phosphorylate the D3 position of the inositol ring of phosphatidylinositol (PI) species (reviewed in Refs. 4–7). PI3Ks are activated through a variety of extracellular stimuli and promote assembly of signaling complexes at the plasma membrane. PI(3,4)P_2 and PI(3,4,5)P_3, products of PI3Ks, recruit specific signaling proteins containing a pleckstrin homology domain that selectively binds 3-phosphoinositides. These signaling proteins include phosphoinositide-dependent kinase 1 (PDK1), Akt, and Vav1 and are involved in a wide range of cellular processes such as cell metabolism, cell cycle progression and survival. PI3K-signaling pathways are counteracted by phosphatase and tensin homologue deleted on chromosome 10 (Pten), a 3-phosphoinositide-specific lipid phosphatase.

The class IA PI3Ks are heterodimeric proteins composed of a catalytic (p110α, p110β, or p110δ) and a regulatory (p85α, p55α, p50α, p85β, or p55γ) subunit and are activated mostly downstream of protein tyrosine kinases (4–7). Each regulatory subunit can interchangeably associate with different catalytic subunits and shows a unique tissue distribution. p85α, a product encoded by the *Pik3r1* gene, is the major regulatory subunit of class IA PI3Ks in most types of cells including immune cells. The *Pik3r1*gene encodes two alternative splicing forms in addition to p85α, p55α, and p50α. Mice lacking p85α alone or mice lacking all products encoded by the *Pik3r1*gene (p85α, p55α, and p50α) show comparable immune phenotypes such that B cell development and activation are impaired while T cell functions are apparently unaffected (8–10). In contrast, mice lacking the p85β regulatory subunit or transgenic (tg) mice

*Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo, Japan; †Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Saitama, Japan; ‡Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; and §Department of Pathology, Research Institute, International Medical Center of Japan, Tokyo, Japan

Received for publication July 12, 2006. Accepted for publication November 16, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Mitsubishi Foundation, a Keio University Special Grant-in-Aid for Innovative Collaborative Research Project, a Grant-in-Aid for Scientific Research for Young Scientist (16790293 to S.M.) from the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research on Priority Areas (14021110 to S.K. and 16043248 to S.M.), a National Grant-in-Aid for the Establishment of a High-Tech Research Center in a private university, and a Scientific Frontier Research Grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

[2] Address correspondence and reprint requests to Dr. Shigeo Koyasu, Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail address: koyasu@sc.itc.keio.ac.jp

[3] Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; tg, transgenic; PI, phosphatidylinositol; Pten, phosphatase and tensin homolog deleted on chromosome 10; PDK, phosphoinositide-dependent kinase; 7AAD, 7-aminoactinomycin D; FTOC, fetal thymic organ culture.

expressing a p85α-binding domain derived from the p110α catalytic subunit show some phenotypes in T cells rather than B cells (11, 12). Among the catalytic subunits of the class IA PI3Ks, inactivation of p110δ, a catalytic subunit expressed predominantly in lymphocytes, impairs Ag-induced proliferation of both T and B cells (13). Interestingly, however, knockout studies have shown that the lack of p110δ leads to defects in the development and function of B cells but not T cells (14, 15).

There has been some indirect evidence suggesting the importance of PI3Ks in early T cell development. For example, T cell-specific deletion of Pten resulted in an increase in thymocyte numbers and allowed transition of DN thymocytes to DP in CD3γ$^{-/-}$ mice (16). Introduction of a constitutively active form of Akt1 along with TCRβ efficiently induced DP cells from Rag-2$^{-/-}$ DN3 cells in an OP9-DL1 culture system (17). Furthermore, reduced expression of PDK1, which functions upstream of Akt, resulted in impaired thymocyte expansion and developmental arrest at DN4 (18). These results strongly suggest the involvement of PI3Ks in T cell development. More recent papers suggested the role of p110γ and p110δ in early thymocyte differentiation (19, 20). To directly elucidate the functions of PI3Ks in β-selection, we used Rag-2$^{-/-}$ mice in which β-selection can be directly examined in vivo by injection of anti-CD3ε mAb. We also assessed the role of p85α in negative as well as positive selection in the thymus using TCR-tg mice on a Rag-2$^{-/-}$ background where no endogenous TCR is involved in the selection procedures. Our results demonstrate here that class IA PI3Ks regulate the β-selection process but not positive and negative selection processes.

Materials and Methods

Mice

p85α$^{-/-}$ mice (21) were backcrossed to BALB/c or C57BL/6 mice for 12 generations before intercrossing heterozygous mice. The colony has been maintained by mating p85α$^{+/-}$ female mice and p85α$^{-/-}$ male mice, because p85α$^{-/-}$ females are virtually sterile. Rag-2$^{-/-}$ mice on a BALB/c background, Rag-2$^{-/-}$ mice on a C57BL/6 background, DO11.10/Rag-2$^{-/-}$ mice on a BALB/c background, Cyt5CC7/Rag-2$^{-/-}$ mice on a B10.A background, P14/Rag-2$^{-/-}$ mice on a C57BL/10 background, and Lck-Cre-tg mice on a C57BL/6 background were obtained from Taconic Farms. p85α$^{-/-}$Rag-2$^{-/-}$ mice were generated by mating p85α$^{-/-}$ and Rag-2$^{-/-}$ mice on a BALB/c background. p85α$^{-/-}$ mice were also crossed with DO11.10/Rag-2$^{-/-}$, Cyt5CC7/Rag-2$^{-/-}$, or P14/Rag-2$^{-/-}$ mice to generate various TCR-tg mice on a p85α$^{-/-}$Rag-2$^{-/-}$ background. Resulting p85α$^{-/-}$DO11.10/Rag-2$^{-/-}$, p85α$^{-/-}$Cyt5CC7/Rag-2$^{-/-}$, and p85α$^{-/-}$P14/Rag-2$^{-/-}$ mice were on BALB/c, B10.A, and C57BL/6 backgrounds, respectively. To generate Rag-2$^{-/-}$ mice with a T cell-specific Pten deficiency, Pten$^{flox/flox}$ mice on a C57BL/6 background (22) were crossed with Rag-2$^{-/-}$ mice on a C57BL/6 background. These mice were further crossed with Lck-Cre-tg mice on a C57BL/6 background. In this paper Lck-Cre/Pten$^{flox/flox}$ mice and Pten$^{flox/flox}$ mice were described as Pten$^{-/-}$ and Pten$^{+/+}$ mice, respectively. All mice were maintained at Taconic Farms or in our animal facility under specific pathogen-free conditions. All experiments were performed in accordance with our institutional guidelines.

In vivo administration of anti-CD3ε mAb and antigenic peptide

Anti-CD3ε mAb (2C11) was immunoaffinity purified from culture supernatant of a hybridoma in our laboratory. A total of 75 μg of 2C11 was delivered by i.p. injection into Rag-2$^{-/-}$ mice to examine the DN to DP transition (23). Sera were prepared from each mouse 2 h after injection and were analyzed for serum Ab titer by ELISA to confirm that the Ab was properly injected. Thymocytes were analyzed at various time points after injection. DO11.10/Rag-2$^{-/-}$ mice were i.v. injected with 50 μg of $OVA_{323–339}$ peptide every 24 h three times. Thymocytes were analyzed 72 h after the first injection.

Flow cytometry and cell sorting

FITC-conjugated anti-CD8α (53-6) and biotinylated anti-CD3ε (2C11) mAbs were produced in our laboratory. FITC-conjugated anti-CD44 (IM7), PE-conjugated anti-CD4 (GK1.5) and anti-CD25 (3C7), allophycocyanin-CyChrome 7 conjugated anti-CD4 (GK1.5), and biotinylated anti-CD2 (RM2-5) mAbs were purchased from BD Biosciences. Biotinylated anti-Vβ2 (B20.6), anti-Vβ3 (KJ25), anti-Vβ7 (TR310), anti-Vβ8 (F23.1), anti-Vβ9 (MR10-2), anti-Vβ12 (MR11-1), anti-Vβ14 (14-2) mAbs were purchased from BD Pharmingen. Binding of biotinylated mAbs was detected with streptavidin-allophycocyanin (BD Biosciences). After incubation with anti-FcγR mAb (2.4G2) to block nonspecific binding of mAbs, cells were stained with the designated Abs in HBSS with 0.1% BSA and were subjected to analysis on a FACSCalibur using the CellQuest program (BD Biosciences). Dead cells were gated out using 7-aminoactinomycin D (7AAD).

To purify DN subsets, DN cells were first enriched by depletion of DP and SP cells using anti-CD8α-coated magnetic beads with an AutoMACS (Miltenyi Biotec). DN cells were then stained with FITC-conjugated anti-CD44, PE-conjugated anti-CD25, allophycocyanin-cychrome-7-conjugated anti-CD4, biotinylated anti-CD3ε mAb, followed by staining with streptavidin-allophycocyanin, and 7AAD. After gating on the CD4 and CD3ε DN population, cells were sorted into DN1–4 populations according to CD44 and CD25 expression profiles on a FACSAria (BD Biosciences). DP ($CD8^+CD4^+$) cells were also isolated using a FACSAria. The sorted populations were reanalyzed on a FACSAria for their purity and were found to be >99% pure.

Western blot analysis

Anti-$p85^{PAN}$ Ab recognizing class IA PI3K regulatory subunits (p85α, p55α, p50α, p85β, and p55γ) was purchased from Upstate Biotechnology. Anti-p110δ (H-119) Ab and anti-Pten (H-19) Ab were purchased from Santa Cruz Biotechnology. Anti-phospho-Akt (Ser^{473}) (193H12) mAb and anti-Akt Ab were purchased from Cell Signaling Technology. Cells were lysed in a lysis buffer solution (1% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 10 mM NaF, 12.5 mM β-glycerophosphate, 1 mM benzamidine, 1% aprotinin, and 2 mM DTT). The cell lysates were applied to SDS-PAGE and were transferred to polyvinyldifluoride membranes to perform Western blot analysis. Reactive proteins were visualized with ECL chemiluminescent substrates (NEN or GE Healthcare Bio-Sciences). To normalize sample loading, membranes were stripped by a stripping buffer solution (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-ME) and subsequently reblotted with anti-ERK2 Ab (Santa Cruz Biotechnology).

Miscellaneous

A p110δ-selective inhibitor IC87114 (24) was provided by ICOS. To confirm the efficacy of IC87114, purified T cells were preincubated for 20 min at 37°C with or without IC87114 and then were stimulated with a mixture of anti-CD3ε and anti-CD28 mAb and goat anti-hamster IgG Ab (MP Biomedicals) (10 μg/ml, respectively) at 37°C for 5 min. Anti-CD28 mAb (37.51) was also immunoaffinity purified from culture supernatant of a hybridoma in our laboratory. T cells were purified from total splenocytes using anti-CD4 and anti-CD8α-coated magnetic beads with an AutoMACS (Miltenyi Biotec) (purity of the cells was >95%).

Fetal thymic organ culture (FTOC) was performed as follows. After dissection of day 14.5 fetuses obtained from timed pregnant mice, individual thymic lobes were placed on a Millipore filter (Whatman) placed on a sponge soaked in RPMI 1640 (Sigma-Aldrich) supplemented with 1% penicillin-streptomycin, 2 mM glutamine, 1% sodium pyruvate, 1% MEM nonessential amino acid, 10 mM HEPES (all from Invitrogen Life Technologies), and 10% FCS (HyClone) in 24-well plates. Plates were incubated at 37°C in 5% CO_2 in air and 95% humidity. After 5 days of incubation, cells were recovered and analyzed.

Statistical analysis was performed with the Mann-Whitney *U* test. Values of $p < 0.05$ were considered significant. Cell numbers and ratios of the thymic population were calculated for independent experiments performed in duplicate or triplicate.

Results

Pre-TCR signal-induced transition of DN to DP thymocytes in Rag-2$^{-/-}$ mice is delayed in the absence of p85α

We examined the role of p85α in early thymocyte development with anti-CD3ε mAb injection into Rag-2$^{-/-}$ mice. Although thymocytes of Rag-2$^{-/-}$ mice are arrested at the DN3 stage due to the lack of TCRβ rearrangement, pre-TCR signals can be generated in Rag-2$^{-/-}$ mice by injection of anti-CD3ε mAb. This mimics β-selection and results in transition from the DN3 through DN4 to DP stage (termed DN3 to DP transition) and cell expansion during such transition (23). To determine whether p85α is involved in the

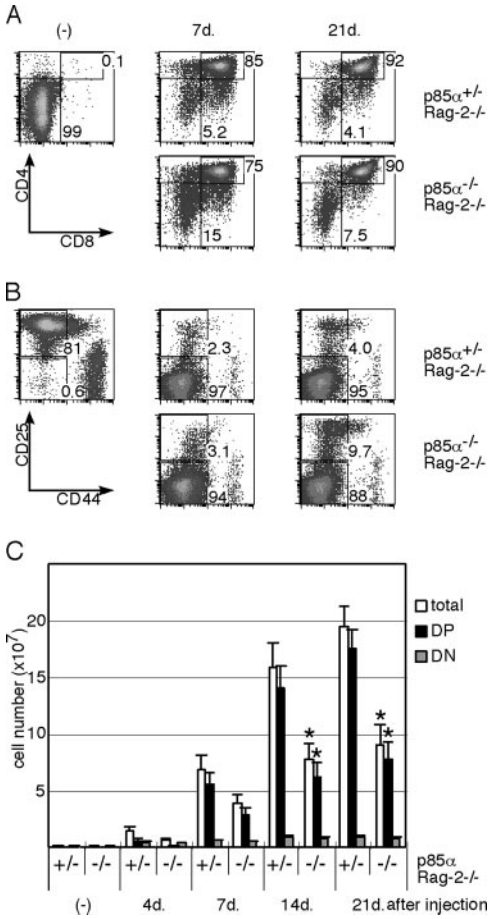

FIGURE 1. DP cell transition upon anti-CD3ε mAb stimulation delays in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice compared with p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice. *A* and *B*, CD8/CD4 and CD44/CD25 surface phenotypes were examined in p85$\alpha^{-/-}$Rag-2$^{-/-}$ and p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice at the indicated times after i.p. injection of anti-CD3ε mAb. Numbers are the percentages of each population. The lack of p85α had no effect on the expression levels of CD3ε on Rag-2$^{-/-}$ thymocytes (data not shown). *C*, Total thymocyte numbers and absolute numbers of DP and DN cells are shown as mean ± SEM in p85$\alpha^{-/-}$Rag-2$^{-/-}$ and p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice before anti-CD3ε mAb stimulation and at the indicated days after injection ($n = 10$–13, except for 4 days where seven mice were analyzed). At 14 and 21 days after injection, thymocyte numbers as well as DP cell numbers in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice are significantly lower than those in p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice (*, $p < 0.01$).

pre-TCR-mediated transition from DN3 to DP, we injected anti-CD3ε mAb into p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice and p85$\alpha^{+/-}$Rag-2$^{-/-}$ littermate controls. Thymocytes of p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice are also arrested at the DN3 stage as observed in Rag-2$^{-/-}$ mice. Anti-CD3ε mAb-induced transition from the DN3 to DP stage was significantly impaired in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice as compared with control mice (Fig. 1). Thymocyte expansion during transition to DP cells was also affected in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice, as total thymocyte numbers as well as DP cell numbers were significantly lower in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice than those of control mice (Fig. 1*C*). These data suggest that while p85α plays a role in the transition from DN3 to DP upon pre-TCR stimulation, it is not essential because this transition still occurs, albeit less efficiently, in p85$\alpha^{-/-}$ cells.

Lack of p85α has little effect on differentiation of DP into CD4/CD8SP thymocytes

Analysis of p85$\alpha^{-/-}$ mice has previously demonstrated that p85$\alpha^{-/-}$ and p85$\alpha^{+/-}$ mice show little difference in the composition of DN, DP and SP thymocytes (8). The apparent lack of difference may be due to some compensatory mechanisms. Even if the PI3K pathway is involved in the TCR signals for positive and negative selection and the lack of p85α affects the signal strength, thymocytes expressing TCRs of different affinities would have substituted wild-type populations. To examine the role of p85α in positive and negative selection processes without such compensatory effects, we analyzed thymic selection using TCR-tg mice on a p85$\alpha^{-/-}$Rag-2$^{-/-}$ background in which no endogenous TCR can be expressed. DO11.10 mice express an H-2^{d}-restricted TCR specific for an $OVA_{323–339}$ peptide. Thymocytes of p85$\alpha^{-/-}$ DO11.10/Rag-2$^{-/-}$ mice develop into CD4 SP cells to a level comparable to p85$\alpha^{+/-}$DO11.10/Rag-2$^{-/-}$ mice (Fig. 2, *A* and *B*, and Table I). Similarly, CD4 SP cell development was unaffected in the absence of p85α in TCR-tg mice expressing another MHC class II-restricted TCR, Cyt5CC7 (Fig. 2*A* and Table I). In addition, CD8 SP cell development appeared normal in the absence of p85α in P14 and OT-I-tg mice expressing MHC class I-restricted TCRs (Fig. 2*A*, Table I and data not shown). These results show that positive selection is unaffected by the lack of p85α. We next investigated the role of p85α in negative selection by in vivo administration of the antigenic peptide, $OVA_{323–339}$, which deletes DP cells in DO11.10 TCR-tg mice (25). As shown in Fig. 2*A* and Table I, no significant difference in the deletion of DP cells was observed between p85$\alpha^{+/-}$ and p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$mice. Furthermore, analysis of TCR Vβ repertoire in thymocyte and splenocyte subpopulations revealed that p85$\alpha^{-/-}$ mice on a BALB/c background had, if any, only slight effect on endogenous superantigen mediated deletion as demonstrated by the deletion of cells expressing Vβ3 and Vβ12 (Fig. 2*C* and data not shown). These results indicate that negative selection is little affected in the absence of p85α as well.

Interestingly, statistics compiled from thymocyte populations showed significant reductions of total as well as DP cell numbers in p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ mice compared with p85$\alpha^{+/-}$ DO11.10/Rag-2$^{-/-}$ mice (Fig. 2*B*). In contrast, DN cell numbers were similar between p85$\alpha^{+/-}$ and p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ mice. Although DP cell numbers were decreased in p85$\alpha^{-/-}$ DO11.10/Rag-2$^{-/-}$ mice, annexin V-positive DP cells were not enriched compared with control mice (data not shown), indicating that cell death is not particularly enhanced in the absence of p85α. Thus, the reduction of DP cell numbers is unlikely due to increased apoptosis mediated by negative selection or death by neglect, but rather reflects the delayed transition from the DN3 to DP stage as shown in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice (Fig. 1). Although CD4 SP cell numbers appeared slightly lower in p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ mice than p85$\alpha^{+/-}$DO11.10/Rag-2$^{-/-}$ mice, it may reflect decreased numbers of p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ DP cell but not the defects during positive selection.

p110δ is decreased in p85$\alpha^{-/-}$ DN cells

The *Pik3r1* gene encodes three alternatively spliced products, p85α, p55α and p50α (4–7). Because our targeting strategy only deletes p85α, potentially leaving the p55α and p50α isoforms intact, the expression levels of these alternative isoforms in p85$\alpha^{-/-}$ cells may differ from those in wild-type cells. As shown previously (10), the expression of p110δ is greatly decreased in p85$\alpha^{-/-}$ B cells (Fig. 3*A*), likely contributing to the observed B cell phenotypes in p85$\alpha^{-/-}$ mice (8, 10). In contrast, DP thymocytes in p85$\alpha^{-/-}$ mice express higher levels of p55α/p50α than those in wild-type mice and expression levels of p110δ were comparable between p85$\alpha^{-/-}$ and p85$\alpha^{+/-}$ mice (Fig. 3*A*).

Since we observed the effect of p85α deficiency only at the DN to DP transition, it was of interest to examine expression levels of PI3K subunits during thymocyte development and to correlate this

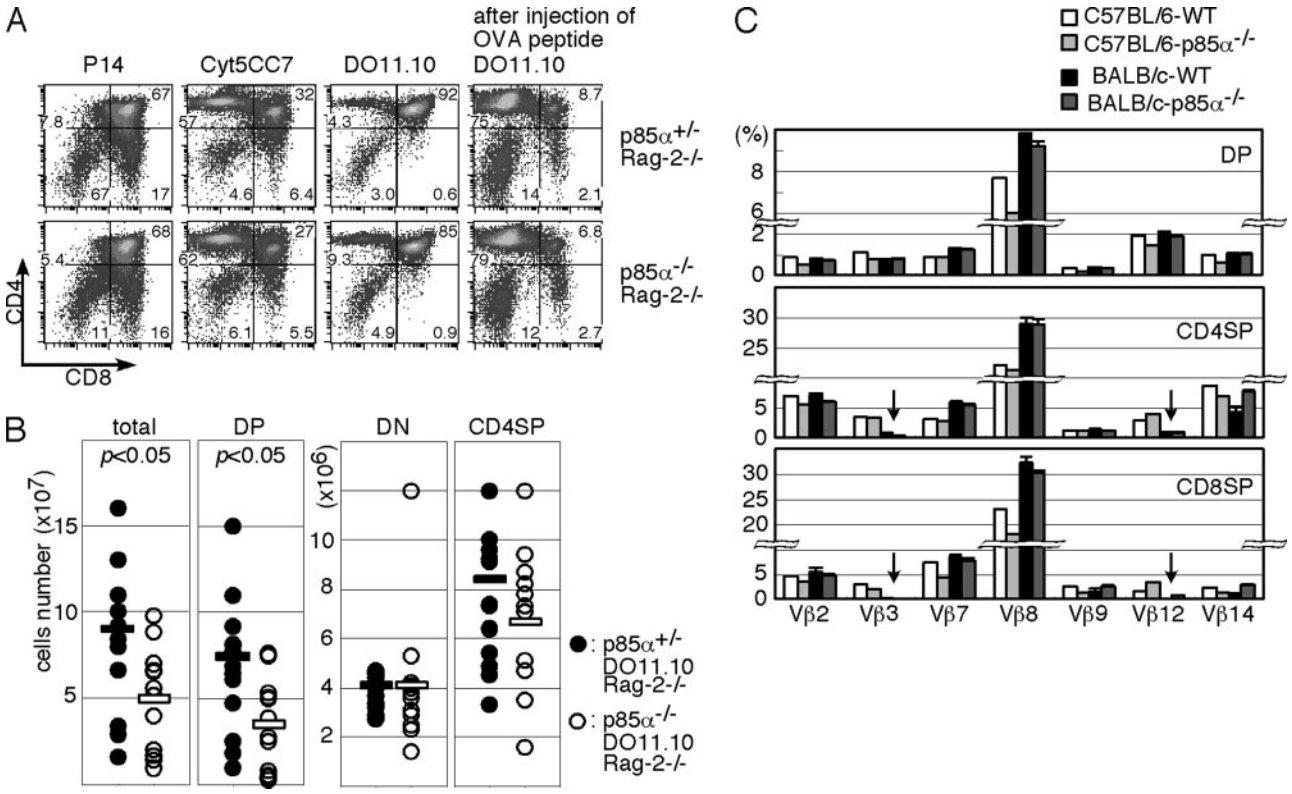

FIGURE 2. The effect of p85α deficiency on thymic selection in TCR-tg mice on a Rag-2$^{-/-}$ background. *A*, CD8/CD4 surface phenotypes were examined in P14, Cyt5CC7, and DO11.10-tg mice on p85$\alpha^{-/-}$Rag-2$^{-/-}$ and p85$\alpha^{+/-}$Rag-2$^{-/-}$ backgrounds. In addition, p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ and control mice were injected with $OVA_{323-339}$ peptide i.v. 72 h before analysis. Numbers are percentages of each population. *A* is representative of two independent experiments. *B*, Analysis of total thymocyte numbers and absolute numbers of DP, DN, and CD4SP cells in p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ mice ($n = 14$) and control mice ($n = 15$). Each circle represents a single mouse and open and bold bars indicate the mean values of p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ and control mice, respectively. Total and DP cell numbers in p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ mice are significantly ($p < 0.05$) lower than those in control mice. *C*, The percentages of CD8SP, CD4SP, and DP thymocytes expressing Vβ2, 3, 4, 7, 8, 9, 12, and 14 were examined in p85$\alpha^{+/+}$ and p85$\alpha^{-/-}$ mice on C57BL/6 and BALB/c backgrounds. Please note that CD8SP and CD4SP cell populations expressing TCR Vβ3 and Vβ12 (indicated by arrows) were efficiently eliminated in both p85$\alpha^{-/-}$ and p85$\alpha^{+/+}$ mice on a BALB/c background.

with thymocyte phenotype. As shown in Fig. 3*B*, expression levels of the p110δ catalytic subunit, the major catalytic subunit expressed in lymphocytes, were significantly lower in thymocytes of p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice than p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice, which are mostly at the DN3 stage. Normalized by the amounts of ERK2, we assessed the reduction of p110δ in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice to 27 ± 16% (mean ± SD, $n = 3$) of p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice. The reduced expression level of p110δ is likely due to the reduction of regulatory subunits including p85α, because association

with regulatory subunits stabilizes the catalytic subunits (4–7). Interestingly, the expression level of p50α was increased after anti-CD3ε mAb injection in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice, and concomitantly the expression level of p110δ in p85$\alpha^{-/-}$Rag-2$^{-/-}$ mice increased to a level similar to that of p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice (Fig. 3*B*).

Table I. *Cell numbers of total, DP, DN, and CD4 or CD8SP thymocyte in TCR-tg mice*[a]

Mice	Total ($\times 10^7$ cells)	DP ($\times 10^7$ cells)	DN ($\times 10^6$ cells)	SP ($\times 10^6$ cells)
P14/Rag-2$^{-/-}$				
p85$\alpha^{+/-}$	8.0 ± 1.0	5.4 ± 0.8	4.8 ± 0.6	6.6 ± 1.6
p85$\alpha^{-/-}$	8.6 ± 2.2	6.5 ± 2.1	5.1 ± 0.7	5.1 ± 0.4
Cyt5CC7/Rag-2$^{-/-}$				
p85$\alpha^{+/-}$	7.0 ± 2.8	1.6 ± 0.9	4.1 ± 1.0	39 ± 17
p85$\alpha^{-/-}$	7.3 ± 1.9	1.5 ± 0.5	4.0 ± 1.3	40 ± 16
DO11.10/Rag-2$^{-/-}$				
p85$\alpha^{+/-}$	9.0 ± 1.4	7.4 ± 1.3	4.1 ± 0.3	8.4 ± 1.2
p85$\alpha^{-/-}$	5.0 ± 0.9[b]	3.5 ± 0.8[b]	4.1 ± 0.8	6.7 ± 0.8
DO11.10/Rag-2$^{-/-}$ (After OVA injection)				
p85$\alpha^{+/-}$	4.9 ± 1.2	1.70 ± 0.64		
p85$\alpha^{-/-}$	2.3 ± 0.2	0.20 ± 0.06		

[a] CD8/CD4 surface phenotypes of each mouse are shown in Fig. 2*A*. Data shown are means ± SEM from more than two independent experiments.

[b] Total and DP cell numbers in p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ mice are significantly ($p < 0.05$) lower than those in control mice.

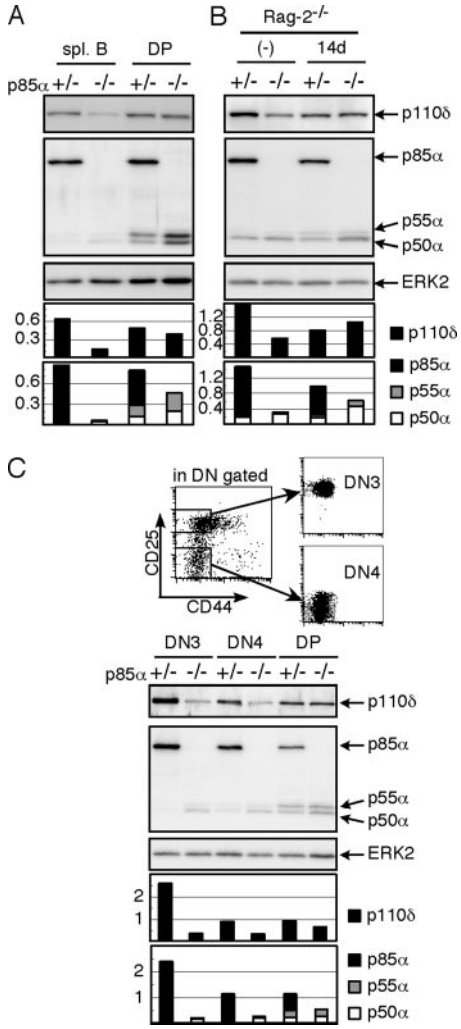

FIGURE 3. Evaluation of expression levels of PI3K subunits by Western blot analysis. *A*, Expression levels of p85α/p55α/p50α and p110δ were examined in splenic B cells and DP thymocytes from p85$\alpha^{-/-}$ and p85$\alpha^{+/-}$ mice. *B*, Expression levels of p85α/p55α/p50α and p110δ were examined in thymocytes from p85$\alpha^{-/-}$Rag-2$^{-/-}$ and p85$\alpha^{+/-}$Rag-2$^{-/-}$ mice without anti-CD3ε mAb injection (−) and at 14 days after injection. *C*, DN3 and DN4 cells were sorted according to CD44/CD25 surface profiles from CD8$^-$CD4$^-$CD3ε$^-$ thymocytes of p85$\alpha^{-/-}$ or p85$\alpha^{+/-}$ mice (*upper panels*). DP cells were also sorted from p85$\alpha^{-/-}$ or p85$\alpha^{+/-}$ mice. Purity was >99% in each population. Expression levels of p85α/p55α/p50α and p110δ were examined in DN3, DN4, and DP cells from p85$\alpha^{-/-}$ mice and p85$\alpha^{+/-}$ mice (*lower panel*). Estimated protein levels of p85α/p55α/p50α and p110δ, which were normalized by the amounts of ERK2, are indicated below.

We next sorted DN3, DN4, and DP cells and examined them for expression levels of PI3K subunits (Fig. 3*C*). The expression level of p110δ in DN3 cells was higher than that in DN4 or DP cells as normalized by ERK2 levels (the ratio of p110δ to ERK2 was 2.4 ± 0.18, 0.94 ± 0.17, and 0.86 ± 0.22 in DN3, DN4 and DP cells, respectively, mean ± SD, $n = 3$). In addition, when we compared expression levels of p110δ between p85$\alpha^{-/-}$ and p85$\alpha^{+/-}$ cells, it was clear that the amount of p110δ was significantly lower in p85$\alpha^{-/-}$ DN3 and DN4 cells than those of heterozygotes. In contrast, p110δ levels in DP cells were not significantly affected by the lack of p85α (Fig. 3*C*). We noted that expression levels of p55α were increased in DP cells and DP cells express higher amounts of p55α/p50α subunits than DN3 and DN4 cells (Fig. 3, *A* and *C*). Such enhanced expression of p55α/p50α likely contributes to the stabilization of p110δ in DP cells, leading to the increase in expression levels of p110δ in the DP cells as compared with DN3 and DN4 p85$\alpha^{-/-}$ cells.

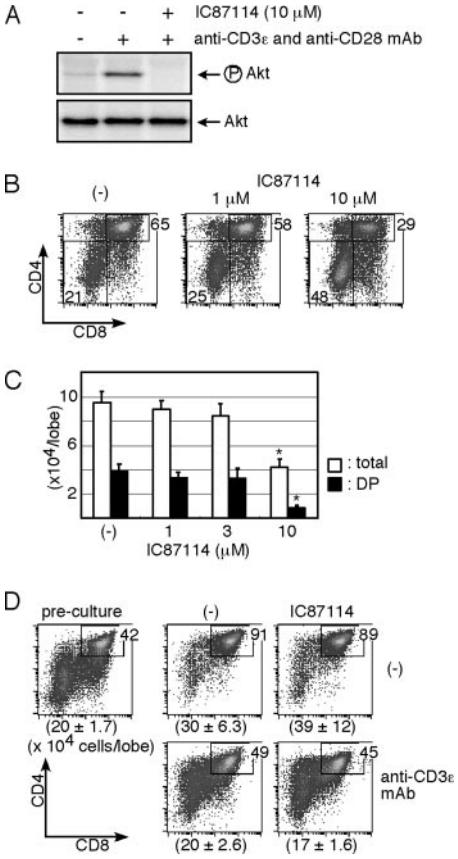

FIGURE 4. The effect of IC87114 on early thymocyte development in E14.5 FTOC. *A*, Splenic T cells derived from wild-type mice were stimulated with anti-CD3ε and anti-CD28 mAb (10 μg/ml each) for 5 min in the presence or absence of 10 μM IC87114. Activation of Akt was evaluated by Western blotting with anti-phospho-Akt mAb. *B*, CD8/CD4 surface phenotypes were examined in E14.5 FTOC cultured with the indicated concentrations of IC87114 for 5 days. *C*, Cell numbers per lobe of total and DP cells are shown as mean ± SEM of four independent experiments. Total and DP cell numbers in fetal thymi cultured with 10 μM IC87114 are significantly lower than untreated lobes (*, $p < 0.01$). *D*, CD8/CD4 surface phenotypes were examined in E17.5 FTOC cultured without (−) or with 10 μg/ml anti-CD3ε mAb in the presence or absence (−) of 10 μM IC87114 for 24 h. Numbers are percentages of DP population. Total cell numbers per lobe were also examined and indicated below (mean ± SEM, $n = 4$).

p110δ is involved in the transition of DN to DP cells in fetal thymocytes

Because our data raise the possibility that p110δ regulates the transition of DN to DP thymocyte upon pre-TCR stimulation, we examined the effect of a selective p110δ inhibitor IC87114 on an early thymocyte development with embryonic day 14.5 (E14.5) FTOC. As has been reported (24), IC87114 blocked Akt phosphorylation, a measure of PI3K activity, in splenic T cells stimulated with anti-CD3ε and anti-CD28 mAb (Fig. 4*A*). Addition of IC87114 in E14.5 FTOC derived from wild-type mice significantly decreased the induction of DP cells in a dose-dependent manner (Fig. 4, *B* and *C*). IC87114 treatment did not induce cell death because cell viability was estimated as 95 ± 5% (mean ± SD) of control culture by 7AAD staining. IC87114 thus blocked pre-TCR induced DN to DP transition without affecting cell viability. In contrast, E17.5 thymocytes, which are at DN4 and DP stages in the

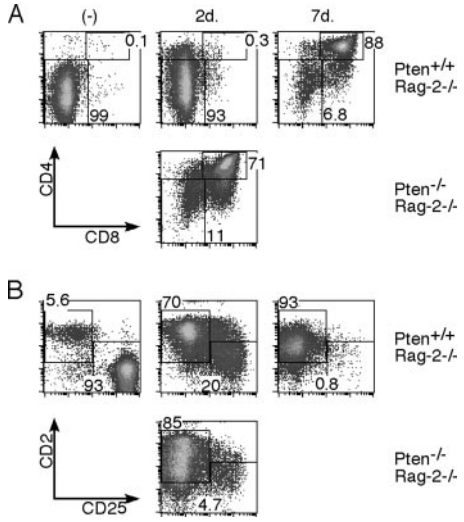

FIGURE 5. DP cell transition is accelerated in Pten$^{-/-}$Rag-2$^{-/-}$ mice compared with Pten$^{+/+}$Rag-2$^{-/-}$ mice upon anti-CD3ε mAb stimulation. *A* and *B*, CD8/CD4 and CD25/CD2 surface phenotypes were examined in Pten$^{-/-}$Rag-2$^{-/-}$ and Pten$^{+/+}$Rag-2$^{-/-}$ mice at the indicated times after injection. Numbers are percentages of DN and DP populations in *A* and CD2^{+}CD25^{-} and CD2^{-}CD25^{+} populations in *B*. Figures are representative of three independent experiments.

beginning of the culture, showed little difference in subsequent SP cell development even in the presence of IC87114 (data not shown). In addition, IC87114 had little effect on DP thymocyte deletion induced by anti-CD3ε mAb in E17.5 FTOC, suggesting that p110δ plays a limited role in negative selection (Fig. 4*D*), which is consistent with the results shown in Fig. 2. These results collectively indicate that p110δ is involved in the pre-TCR signal during DN to DP transition but not in subsequent transition to SP thymocytes in both adult and fetal thymus.

Loss of Pten accelerates transition of DN to DP cells

Generation of PI(3,4)P_2 and PI(3,4,5)P_3 by PI3Ks is reversible and is opposed by a specific lipid phosphatase, termed Pten. It is known that the absence of Pten causes sustained PI3K signaling. To further confirm that lipid products generated by PI3Ks are involved in transition of DN to DP cells, we crossed Pten$^{flox/flox}$ mice, Rag-2$^{-/-}$ mice and Lck-Cre-tg mice to generate Rag2$^{-/-}$ mice with T cell specific deletion of Pten. Lck-Cre/Pten$^{flox/flox}$ mice and Pten$^{flox/flox}$ mice were described here as Pten$^{-/-}$ and Pten$^{+/+}$ mice, respectively. Pten deficiency had no effect on expression levels of p85α and p110δ in thymocytes on a Rag-2$^{-/-}$ background (data not shown). We then injected anti-CD3ε mAb into Pten$^{-/-}$Rag-2$^{-/-}$ and Pten$^{+/+}$Rag-2$^{-/-}$ mice. Without anti-CD3ε mAb stimulation, most Pten$^{-/-}$Rag-2$^{-/-}$ thymocytes remain at the DN3 stage as observed in Rag-2$^{-/-}$ mice. However, two of seven mice spontaneously developed DP cells and such DP cells showed an unusual CD2^{+}CD25^{+} phenotype (data not shown). Mice with such DP cells were also found after anti-CD3ε mAb injection. We thus excluded mice with such unusual CD2^{+}CD25^{+} DP cells from further analyses because those mice would have had such DP cells even before anti-CD3ε mAb injection. CD25 is down-regulated and CD2 up-regulated upon β-selection and CD2^{-}CD25^{+} Rag-2$^{-/-}$ DN3 cells become CD2^{+}CD25^{-} cells immediately after anti-CD3ε mAb stimulation before reaching the DP stage (Ref. 23 and Fig. 5*B*). Two days after anti-CD3ε mAb injection, CD2^{+}CD25^{-} DP cells were observed in Pten$^{-/-}$Rag-2$^{-/-}$ mice while CD2 was induced in most of the cells without transition into DP cells in Pten$^{+/+}$Rag-2$^{-/-}$ mice (Fig. 5). As demonstrated in Fig. 1, these cells became DP cells 7 days after anti-CD3ε mAb injection. Moreover, total thymocyte numbers at 2 days after injection were higher in Pten$^{-/-}$Rag-2$^{-/-}$ mice than Pten$^{+/+}$Rag-2$^{-/-}$ mice ($99 \pm 28 \times 10^6$, $n = 4$ vs $11 \pm 4 \times 10^6$, $n = 5$, respectively). It should be noted that expression profiles of developmental markers such as CD8/CD4 and CD25/CD2 (Fig. 5) as well as thymocyte numbers (data not shown) in Pten$^{-/-}$Rag-2$^{-/-}$ mice at 2 days after anti-CD3ε mAb injection were quite similar to those in Pten$^{+/+}$Rag-2$^{-/-}$ mice at 7 days after injection. These data indicate that the loss of Pten dramatically accelerates transition of DN3 to DP cells and that the lipid products generated by PI3Ks are indeed involved in pre-TCR signals.

Discussion

Initial studies on p85α-deficient mice have shown that development and function of B cells are impaired but those of T cells are grossly normal (8, 9). As demonstrated here, when we focused on the β-selection process using the unique system by injecting anti-CD3ε mAb into Rag-2$^{-/-}$ mice, it was revealed that the lack of p85α delayed transition of DN to DP cells. Previous studies have shown that the proliferation of peripheral T cells is attenuated in mice expressing an inactive form of p110δ or Akt (13, 26), suggesting that the PI3K/Akt pathway modulate TCR signals. If this is also the case in the thymus, positive and negative selection would be affected by the lack of p85α that altered the TCR repertoire selection but total cell numbers after selection may not be different between p85$\alpha^{+/-}$ and p85$\alpha^{-/-}$ mice. Accordingly, the p85α deficiency phenotype may have been hidden in previous studies. Indeed, p85$\alpha^{-/-}$DO11.10/Rag-2$^{-/-}$ mice expressing a defined TCR with no endogenous TCRs showed a decrease in DP cell numbers compared with control mice. Furthermore, a p110δ-selective inhibitor, IC87114, suppressed development of DP cells in E14.5 FTOC after 5 days in culture, indicating that p110δ is involved in the transition of DN to DP cells in fetal thymus as well. Interestingly, p85α deficiency had little effect on DP cell numbers in some TCR-tg mice such as Cyt5CC7, P14, and OT-I on a Rag-2$^{-/-}$ background. These differences could be explained by the fact that the expression level of TCR in each tg mouse varies according to promoter and copy numbers of transgene. In addition, the timing of transgene expression varies in different tg mice. Thus, a higher expression level of TCRβ in DN3 might generate strong signal enough to overcome the attenuation of DN to DP transition caused by lack of p85α. In contrast to the effect of the DN to DP transition, positive and negative selection was unaffected in the absence of p85α. We noted that expression levels of p110δ in p85$\alpha^{-/-}$ DP and SP cells were comparable to those of wild-type counterparts, likely due to increased expression of p55α/p50α. Although the lack of p85α would also affect the expression level of p110α expressed in thymocytes (12), we were not able to examine the amounts of p110α in p85$\alpha^{-/-}$ thymocytes due to lack of an appropriate Ab. However, immunoprecipitation of p110δ from total thymocyte lysates resulted in nearly complete depletion of p85α/p55α/p50α (our unpublished observations), indicating that p110δ is indeed the major catalytic subunit and the contribution of p110α must be marginal, if any. Expression levels of p110δ in wild-type DN3 cells were higher than those in DN4 or DP cells (Fig. 3*C*), suggesting an important role for p110δ in β-selection rather than thymic selection at DP stage. Indeed, when we analyzed E14.5 FTOC for 7 and 9 days or E17.5 FTOC for 5 days, no defect in subsequence development of SP cells was observed in the presence of IC87114 (our unpublished observations). Similarly, a previous

study also reported that the inhibition of p110δ function by IC87114 had little effect in FTOC in 7-day cultures (20).

Our observation is consistent with a report demonstrating that mice with a reduced expression level of PDK1, which functions downstream of PI3Ks, as well as mice with T cell specific deletion of PDK1 show impaired expansion of thymocytes (18). Mice deficient for p110γ, the catalytic subunits of class IB PI3K, have decreased thymocyte numbers compared with wild type (27). Although the substrate specificity of class IB PI3K which consists of a catalytic subunit p110γ and a regulatory subunit p101 is similar to that of class IA PI3K, class IB PI3K is activated by G-protein-coupled receptors including chemokine receptors. Interestingly, it was shown that introduction of a constitutively active mutant of class IA PI3K was able to restore the defect of p110$\gamma^{-/-}$ mice (28). Rodríguez-Borlado et al. (28) have reported that forced expression of a mutant form of p85α leading to activation of catalytic subunits resulted in acceleration of CD4 SP cell differentiation without affecting CD8 SP cells. In contrast, tg mice established by Barbee and Alberola-Ila (12) that express a portion of p110α subunit that lacks catalytic activity but binds p85α, leading to activation of the endogenous catalytic subunit showed no difference in CD4/CD8 commitment. Our results are consistent with the latter report and there was no difference in differentiation to CD4 SP and CD8 SP cells. However, these tg mice show no difference in DN or DP populations compared with non-tg controls. The reason for such discrepancy is unknown at the moment but difference in TCR repertoire may have compensated for the defects as discussed above, because mice used in previous studies were not on a Rag2$^{-/-}$ background. Interestingly, a recent report has shown that thymocyte numbers are significantly lower in p110$\gamma^{-/-}$p110$\delta^{-/-}$ double knockout mice than individual single-deficient mice, likely due to not only facilitation of apoptosis in DP cells but also defects in β-selection (19, 20). The more severe phenotype of p110$\gamma^{-/-}$ p110$\delta^{-/-}$ mice likely reflects the fact that the PI3K/Akt-signaling pathway via various receptors synergistically regulates thymocyte development.

In contrast to the deficiency of PI3Ks, the absence of Pten activates the PI3K/Akt-signaling pathway. The loss of Pten dramatically accelerated transition of DN to DP thymocytes and cellular expansion. These observations are consistent with our conclusion that the PI3K/Akt-signaling pathway is involved in pre-TCR signals during the DN to DP transition. As discussed above, thymocytes of two of seven Pten$^{-/-}$Rag-2$^{-/-}$ mice spontaneously developed into DP cells at 9 wk of age without anti-CD3ε mAb injection. Such spontaneous development of DP cells was independent of the age (our unpublished observations) and such DP cells showed an abnormal phenotype as they express both CD2 and CD25. The loss of Pten is often associated with tumorigenesis (29) and it is possible that the loss of Pten leads to thymoma generation. In the case of T cell-specific deletion of Pten in mice, the first clinical signs of tumor formation were observed in mice at 6–7 wk and all mice died within 17 wk (22). The observed variability of the thymocyte phenotype of Pten$^{-/-}$ Rag-2$^{-/-}$ mice may be ascribed to the timing of Cre expression. Although the p56^{lck} proximal promoter used here is known to be active in DN1 cells, expression of Pten protein was still detectable in the Pten$^{-/-}$Rag-2$^{-/-}$ thymus (our unpublished observations) where thymocytes are arrested at the DN3 stage. Perhaps the p56^{lck} proximal promoter exhibits altered expression patterns as a transgene. However, anti-CD3ε mAb injection led to complete loss of Pten expression (data not shown), likely due to enhanced activity of p56^{lck} proximal promoter.

The PI3K/Akt-signaling pathway is known to suppress apoptosis, increase cell size due to activation of cellular metabolism, and induce cell cycle progression through expression of cyclins and cytokines (30–32). The levels of BrdU uptake in thymocytes in p85$\alpha^{-/-}$ mice were comparable with those in p85$\alpha^{+/-}$ mice (data not shown), suggesting that proliferation during transition from the DN to DP stages was not affected dramatically by the lack of p85α, although it is possible that slight difference in cell cycle progression leads to difference in DP cell numbers. Additionally, IC87114 suppressed DP cell induction without cell death in E14.5 FTOC, indicating that p110δ is not critical for survival at least at the DN stage. In our preliminary experiments, inhibition of mammalian target of rapamycin, a downstream molecule of the PI3K/Akt-signaling pathway, by rapamycin resulted in blockade of DN to DP transition in E14.5 FTOC (our unpublished observations), raising the possibility that the PI3K/Akt-signaling pathway regulates the β-selection through mammalian target of rapamycin. Future studies with mice where the PI3K/Akt-signaling pathway is perturbed should reveal the molecular mechanisms underlying transition of DN to DP thymocytes.

Acknowledgments

We are grateful to Dr. Akira Suzuki of Akita University and Dr. Tak W. Mak of the Ontario Cancer Institute for providing us with the Pten^{flox} mice. We thank laboratory members for their fruitful discussion and Dr. Linda K. Clayton for critical reading of the manuscript and valuable suggestions. Thank are also due to Drs. C. Sadhu and J. Hayflick of ICOS Corporation for the generous gifts of IC87114.

Disclosures

The authors have no financial conflict of interest.

References

1. Starr, T. K., S. C. Jameson, and K. A. Hogquist. 2003. Positive and negative selection of T cells. *Annu. Rev. Immunol.* 21: 139–176.
2. Michie, A. M., and J. C. Zúñiga-Pflücker. 2002. Regulation of thymocyte differentiation: pre-TCR signals and β-selection. *Semin. Immunol*. 14: 311–323.
3. Gill, J., M. Malin, J. Sutherland, D. Gray, G. Hollander, and R. Boyd. 2003. Thymic generation and regeneration. *Immunol. Rev.* 195: 28–50.
4. Koyasu, S. 2003. The role of PI3K in immune cells. *Nat. Immunol.* 4: 313–319.
5. Okkenhaug, K., and B. Vanhaesebroeck. 2003. PI3K in lymphocyte development, differentiation and activation. *Nat. Rev. Immunol.* 3: 317–330.
6. Deane, J. A., and D. A. Fruman. 2004. Phosphoinositide 3-kinase: diverse roles in immune cell activation. *Annu. Rev. Immunol.* 22: 563–598.
7. Vanhaesebroeck, B., K. Ali, A. Bilancio, B. Geering, and L. C. Foukas. 2005. Signalling by PI3K isoforms: insights from gene-targeted mice. *Trends Biochem. Sci.* 30: 194–204.
8. 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 p85α subunit of phosphoinositide 3-kinase. *Science* 283: 390–392.
9. 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 p85α. *Science* 283: 393–397.
10. Suzuki, H., S. Matsuda, Y. Terauchi, M. Fujiwara, T. Ohteki, T. Asano, T. W. Behrens, T. Kouro, K. Takatsu, T. Kadowaki, and S. Koyasu. 2003. PI3K and Btk differentially regulate B cell antigen receptor-mediated signal transduction. *Nat. Immunol.* 4: 280–286.
11. Deane, J. A., M. J. Trifilo, C. M. Yballe, S. Choi, T. E. Lane, and D. A. Fruman. 2004. Enhanced T cell proliferation in mice lacking the p85β subunit of phosphoinositide 3-kinase. *J. Immunol.* 172: 6615–6625.
12. Barbee, S. D., and J. Alberola-Ila. 2005. Phosphatidylinositol 3-kinase regulates thymic exit. *J. Immunol.* 174: 1230–1238.
13. Okkenhaug, K., A. Bilancio, G. Farjot, H. Priddle, S. Sancho, E. Peskett, W. Pearce, S. E. Meek, A. Salpekar, M. D. Waterfield, et al. 2002. Impaired B and T cell antigen receptor signaling in p110δ PI 3-kinase mutant mice. *Science* 297: 1031–1034.
14. Clayton, E., G. Bardi, S. E. Bell, D. Chantry, C. P. Downes, A. Gray, L. A. Humphries, D. Rawlings, H. Reynolds, E. Vigorito, and M. Turner. 2002. A crucial role for the p110δ subunit of phosphatidylinositol 3-kinase in B cell development and activation. *J. Exp. Med.* 196: 753–763.
15. Jou, S. T., N. Carpino, Y. Takahashi, R. Piekorz, J. R. Chao, N. Carpino, D. Wang, and J. N. Ihle. 2002. Essential, nonredundant role for the phosphoinositide 3-kinase p110δ in signaling by the B-cell receptor complex. *Mol. Cell Biol.* 22: 8580–8591.
16. Hagenbeek, T. J., M. Naspetti, F. Malergue, F. Garçon, J. A. Nunès, K. B. Cleutjens, J. Trapman, P. Krimpenfort, and H. Spits. 2004. The loss of PTEN allows TCR αβ lineage thymocytes to bypass IL-7 and pre-TCR-mediated signaling. *J. Exp. Med.* 200: 883–894.

17. Ciofani, M., and J. C. Zúñiga-Pflücker. 2005. Notch promotes survival of pre-T cells at the β-selection checkpoint by regulating cellular metabolism. *Nat. Immunol.* 6: 881–888.
18. Hinton, H. J., D. R. Alessi, and D. A. Cantrell. 2004. The serine kinase phosphoinositide-dependent kinase 1 (PDK1) regulates T cell development. *Nat. Immunol.* 5: 539–545.
19. Webb, L. M., E. Vigorito, M. P. Wymann, E. Hirsch, and M. Turner. 2005. Cutting edge: T cell development requires the combined activities of the p110γ and p110δ catalytic isoforms of phosphatidylinositol 3-kinase. *J. Immunol.* 175: 2783–2787.
20. Swat, W., V. Montgrain, T. A. Doggett, J. Douangpanya, K. Puri, W. Vermi, and T. G. Diacovo. 2006. Essential role of PI3Kδ and PI3Kγ in thymocyte survival. *Blood* 107: 2415–2422.
21. Terauchi, Y., Y. Tsuji, S. Satoh, H. Minoura, K. Murakami, A. Okuno, K. Inukai, T. Asano, Y. Kaburagi, K. Ueki, et al. 1999. Increased insulin sensitivity and hypoglycaemia in mice lacking the p85α subunit of phosphoinositide 3-kinase. *Nat. Genet.* 21: 230.
22. Suzuki, A., M. T. Yamaguchi, T. Ohteki, T. Sasaki, T. Kaisho, Y. Kimura, R. Yoshida, A. Wakeham, T. Higuchi, M. Fukumoto, et al. 2001. T cell-specific loss of Pten leads to defects in central and peripheral tolerance. *Immunity* 14: 523–534.
23. Shinkai, Y., and F. W. Alt. 1994. CD3ε-mediated signals rescue the development of $CD4^+CD8^+$ thymocytes in RAG-$2^{-/-}$ mice in the absence of TCR β chain expression. *Int. Immunol.* 6: 995–1001.
24. Sadhu, C., B. Masinovsky, K. Dick, C. G. Sowell, and D. E. Staunton. 2003. Essential role of phosphoinositide 3-kinase δ in neutrophil directional movement. *J. Immunol.* 170: 2647–2654.
25. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes in vivo. *Science* 250: 1720–1723.
26. Arimura, Y., F. Shiroki, S. Kuwahara, H. Kato, U. Dianzani, T. Uchiyama, and J. Yagi. 2004. Akt is a neutral amplifier for Th cell differentiation. *J. Biol. Chem.* 279: 11408–11416.
27. Sasaki, T., J. Irie-Sasaki, R. G. Jones, A. J. Oliveira-dos-Santos, W. L. Stanford, B. Bolon, A. Wakeham, A. Itie, D. Bouchard, I. Kozieradzki, et al. 2000. Function of PI3Kγ in thymocyte development, T cell activation, and neutrophil migration. *Science* 287: 1040–1046.
28. Rodríguez-Borlado, L., D. F. Barber, C. Hernández, M. A. Rodríguez-Marcos, A. Sánchez, E. Hirsch, M. Wymann, C. Martínez-A, and A. C. Carrera. 2003. Phosphatidylinositol 3-kinase regulates the CD4/CD8 T cell differentiation ratio. *J. Immunol.* 170: 4475–4482.
29. Suzuki, A., J. L. de la Pompa, V. Stambolic, A. J. Elia, T. Sasaki, I. del Barco Barrantes, A. Ho, A. Wakeham, A. Itie, W. Khoo, et al. 1998. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr. Biol.* 8: 1169–1178.
30. Ward, S. G., and D. A. Cantrell. 2001. Phosphoinositide 3-kinases in T lymphocyte activation. *Curr. Opin. Immunol.* 13: 332–338.
31. Wymann, M. P., M. Zvelebil, and M. Laffargue. 2003. Phosphoinositide 3-kinase signalling—which way to target? *Trends Pharmacol. Sci.* 24: 366–376.
32. Fruman, D. A. 2004. Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling. *Curr. Opin. Immunol.* 16: 314–320.