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Phosphatidylinositol 3-Kinase Regulates the CD4/CD8 T Cell Differentiation Ratio[1]

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The signaling pathways that control T cell differentiation have only begun to be elucidated. Using T cell lines, it has been shown that class IA phosphatidylinositol 3-kinase (PI3K), a heterodimer composed of a p85 regulatory and a p110 catalytic subunit, is activated after TCR stimulation. Nonetheless, the contribution of p85/p110 PI3K isoforms in T cell development has not been described. Mice deficient in the other family of class I PI3K, p110γ, which is regulated by G protein-coupled receptors, exhibit reduced thymus size. Here we examine T cell development in p110γ-deficient mice and in mice expressing an activating mutation of the p85 regulatory subunit, p65^{PI3K}, in T cells. We show that p110γ-deficient mice have a partial defect in pre-TCR-dependent differentiation, which is restored after expression of the p65^{PI3K} activating mutation. Genetic alteration of both PI3K isoforms also affects positive selection; p110γ deletion decreased and p65^{PI3K} expression augmented the CD4$^+$/CD8$^+$ differentiation ratio. Finally, data are presented showing that both PI3K isoforms influenced mature thymocyte migration to the periphery. These observations underscore the contribution of PI3K in T cell development, as well as its implication in determining the CD4$^+$/CD8$^+$ T cell differentiation ratio in vivo. *The Journal of Immunology,* **2003, 170: 4475–4482.**

T cell maturation begins in the thymic cortex, where most immature progenitors—cells that lack CD4 and CD8 molecule expression (CD4$^-$CD8$^-$ double negative (DN)[4])—are found. These progenitors can be further subdivided into different stages based on CD44 and CD25 expression. The earliest progenitors are CD44$^+$CD25$^-$ (DN stage I), followed by CD44$^+$CD25$^+$, CD44$^-$CD25$^+$, and CD44$^-$CD25$^-$ populations (stages II–IV, respectively). At the CD44$^-$CD25$^+$ stage (DN III), cells pass the first T cell differentiation checkpoint. Cells that successfully reorganize the TCR β locus and express a functional receptor complex (known as pre-TCR) proliferate, down-regulate CD25, and differentiate into CD4$^+$CD8$^+$ double-positive (DP) cells. Assembly of the TCRα-chain in these cells leads to expression of a functional TCR$\alpha\beta$, which permits T cells to pass to the second checkpoint. Cells expressing a TCR that recognizes self Ags are eliminated (negative selection), whereas those whose TCR recognizes the appropriate MHC molecules survive and differentiate into mature single-positive CD4$^+$ or CD8$^+$ cells (positive selection; reviewed in Refs. 1 and 2).

The intracellular signaling cascades that govern T cell differentiation have begun to be elucidated. In early stages of DN cell differentiation, IL-7R-derived signals as well as Rho GTPase (3, 4) appear essential for driving T cell development. The contribution of the Src-Tyr kinase Lck at the pre-TCR-dependent stage of DN cell differentiation is essential for cells to proceed to the DP differentiation stage (5). Positive selection involves TCR stimulation and requires Lck, Zap 70, mitogen-activated protein kinase (MAPK), and Vav activation (1, 6–11). How signals that induce positive selection trigger differentiation into the CD4$^+$ or CD8$^+$ mature cell type is not fully understood. Nonetheless, quantitative differences in MAPK and Lck activation appear to alter the CD4$^+$/CD8$^+$ differentiation ratio (12, 13).

The class I phosphatidylinositol (PI) 3-kinases (PI3Ks) are heterodimeric proteins composed of a regulatory and a catalytic subunit that induce rapid, transient formation of PI-3,4-P_2 and PI-3,4,5-P_3. Class IB PI3K is composed of the p101 regulatory and the p110γ catalytic subunits and is activated after G protein-coupled receptor (GPCR) stimulation. Class IA PI3K comprises a regulatory (p85α, β, or p55γ) and a p110 catalytic (α, β, or δ) subunit and is activated by Tyr kinase receptor stimulation (reviewed in Ref. 14). PI3K is essential for B cell differentiation (15, 16), but although it is activated by the TCR (17), the role of PI3K in T cell differentiation remains unclear. In fact, p110$\alpha^{-/-}$ and p110$\beta^{-/-}$ mice exhibit embryonic lethality (18, 19), and p85$\alpha^{-/-}$ mice have no obvious defects in T cell populations, but they still express p85β and p55γ (15, 16). The p110δ isoform was recently shown to be essential for mature B and T cell activation, but it does not seem to affect T cell development (20). Finally, p110$\gamma^{-/-}$ mice show a

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[4] Abbreviations used in this paper: DN, double negative; DP, double positive; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PI3K, PI3-kinase; GPCR; G protein-coupled receptor; Tg, transgenic; HSA, heat-stable Ag; Wt, wild type; PKB, protein kinase B.

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defect in thymus size (21), although the details of how this isoform controls thymic development are presently unknown.

We performed a detailed phenotypic analysis of T cell development in mice deficient in class IB PI3K (p110γ). In addition, to examine the role of the p85/p110 PI3K isoforms (class IA) in T cell development, we analyzed the phenotype of mice expressing an activating mutation of the p85 regulatory subunit, p65^{PI3K}, in T cells. This mutant regulatory subunit associates with the p110 catalytic subunit, moderately increasing basal PI-3,4,5-P_3 levels and significantly enhancing the transient PI3K activation that follows receptor activation (22, 23). p65^{PI3K} potentially acts on all three class IA catalytic subunits, constituting a useful tool for the study of the role of class IA p85/p110 PI3K isoforms in T cell development.

We found that p110γ deficiency partially impaired DN-to-DP transition and diminished the proliferative expansion that accompanies development from the DN to the DP stage (thymus growth). In support of a role for PI3K at this stage, p65^{PI3K} expression increased the pre-TCR-dependent DN-to-DP transition, although it did not affect thymus growth. At the positive selection stage, p110γ deficiency reduced the CD4$^+$/CD8$^+$ T cell differentiation ratio, whereas p65^{PI3K} expression augmented this ratio. Moreover, p65^{PI3K} expression corrected p110$\gamma^{-/-}$ mouse differentiation defects, except for thymus growth. These results illustrate that PI3K modulates T cell differentiation at several stages. The p110γ isoform regulates thymic growth. In addition, p110γ influences the pre-TCR-dependent DN-to-DP cell transition and affects the CD4/CD8 T cell differentiation ratio. Enhanced class IA p85/p110 PI3K activation affects the same differentiation steps, except for thymus growth, suggesting that class IA PI3K activation may also contribute to regulation of T cell development. The results presented are the first demonstration of the role of p110γ in the signaling pathways that regulate pre-TCR-dependent differentiation and in CD4$^+$/CD8$^+$ lineage commitment during positive selection.

Materials and Methods

Mice

p65^{PI3K} transgenic (Tg) mice were generated as described (24). The Tg colony was maintained by crosses with C57BL/6 females, and offspring were analyzed by PCR 30 days after birth. The p110γ null mice were previously described and maintained in heterozygosis (25). The 5CC7 TCR (Vβ3Vα11) and F5 TCR (Vβ11Vα4) Tg mice were previously described (26, 27) and were kindly provided by Drs. M. Davis (Howard Hughes Medical Institute, Stanford, CA) and D. Kioussis (Medical Research Council, London, U.K.), respectively. 5CC7 TCR Tg mice were crossed with p110γ null mice (on the C57BL/6 background) and with p65^{PI3K} Tg mice. F5 TCR Tg mice were also crossed with p110γ null mice. Offspring were analyzed by PCR as described (24) and by flow cytometry to verify the appropriate TCR and MHC. All mice were bred and maintained under specific pathogen-free conditions at the National Center for Biotechnology animal facility.

Flow cytometry analysis, BrdU labeling, and intrathymic FITC injection

Thymus, spleen, and lymph node cell suspensions were prepared by grinding tissue through sterile wire mesh. Cells were counted by trypan blue exclusion and erythrocytes were lysed with a hypotonic ammonium chloride solution. For cell surface staining, all Abs used were FITC-, PE-, or biotin-conjugated, and cells were stained with saturating Ab concentrations at 4°C. Biotinylated Abs were developed with streptavidin-spectral red (Southern Biotechnology, Birmingham, AL). The Abs used were heat-stable Ag (HSA; CD24, M1/69), CD8 (Ly-2, 53-6, 7), CD4 (L3T4, H129.19), CD3 (145-2C11), CD25 (IL-2R α-chain), CD44 (pgp1, IM7), Vβ3 (KJ25), Vβ11(RR3-15), Vα11 (RR8-1), I-Ek (17-3-3), and H-2b (AF6-88.5), all from BD PharMingen (San Diego, CA). TCR$\alpha\beta$ Ab (H57-597) was from Southern Biotechnology, and anti-BrdU Ab was from BD Biosciences (San Jose, CA). BrdU labeling and analysis were performed as described (28). Briefly, BrdU was administrated for 8 days in drinking water to wild type (Wt) and p65^{PI3K} Tg mice, and then the presence of spleen T cells incorporating BrdU^{low} was examined by flow cytometry. Intrathymic FITC injections and analysis of recent thymic emigrants were as described (29, 30). Cells were analyzed on an EPICS XL using System II software (Coulter, Miami, FL).

Results

Enhanced class IA p85/p110 PI3K activation regulates DN cell differentiation

We previously reported that young mice expressing the p65^{PI3K} transgene in T cells exhibited a 30% increase in the proportion of CD4$^+$ peripheral T cells. Mice remain healthy for 1 year; however, at ~12–15 mo of age, p65^{PI3K} induces development of a lymphoproliferative disease caused by the enhanced memory cell survival (24). This enhanced memory cell survival in adult mice does not explain the 30% increase in CD4$^+$ T cells in young mice. We postulated that p65^{PI3K} may affect thymic development. Here we examine T cell differentiation in young (5- to 8-wk-old) p65^{PI3K} Tg mice.

Thymic cellularity was similar in Wt and Tg littermates (data not shown). Nonetheless, the percentage of DN cells was reduced in Tg compared with Wt mice (Fig. 1*A*; Student's *t* test; $p = 0.0001$). This represented a systematic reduction in absolute DN cell numbers from a mean of $7.4 \pm 2.9 \times 10^6$ (Wt thymus) to $4.4 \pm 2.2 \times 10^6$ (Tg thymus). Examination of DN differentiation steps (31) showed a significant decrease in the percentage of CD44$^-$CD25$^+$ cells (DN stage III) in p65^{PI3K} Tg compared with Wt mice (Fig. 1*B*; $p = 0.002$) and no significant changes in the remaining populations. The percentage of DP cells was moderately increased in Tg mice (Fig. 1*C*; $p = 0.0008$). The p65^{PI3K} Tg mice thus exhibit reduced DN cell numbers, a lower proportion of cells at the CD44$^-$CD25$^+$ stage, and a moderately increased percentage of DP cells.

p65^{PI3K} increases CD4$^+$ T cell generation

We next examined mature thymic T cell populations. The p65^{PI3K} Tg mouse thymuses had lower percentages of CD4$^+$ cells compared with Wt animals (Fig. 2*A*; $p = 0.007$), with no significant

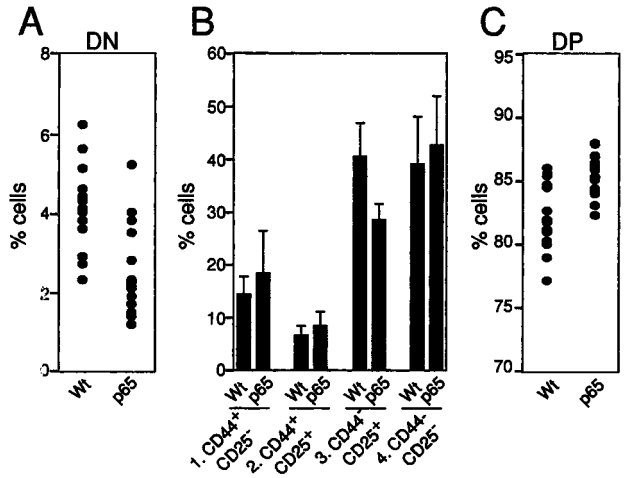

FIGURE 1. Enhanced class IA PI3K activation increases DN cell differentiation. *A*, Thymocytes from 5- to 8-wk-old p65^{PI3K} Tg or Wt littermates were stained with anti-CD4 and -CD8 mAb, and the percentage of DN cells was analyzed by flow cytometry. Each dot represents a single mouse. *B*, Thymocytes from p65^{PI3K} Tg or Wt littermates were stained simultaneously with anti-CD4, -CD8, -CD44, and -CD25 mAbs. The CD4$^-$CD8$^-$ (DN) population was gated, and the percentage of the four indicated populations was examined. The mean ± SD from 12 mice examined is represented. *C*, Cells were processed as in *A*, and the percentage of DP cells was analyzed by flow cytometry.

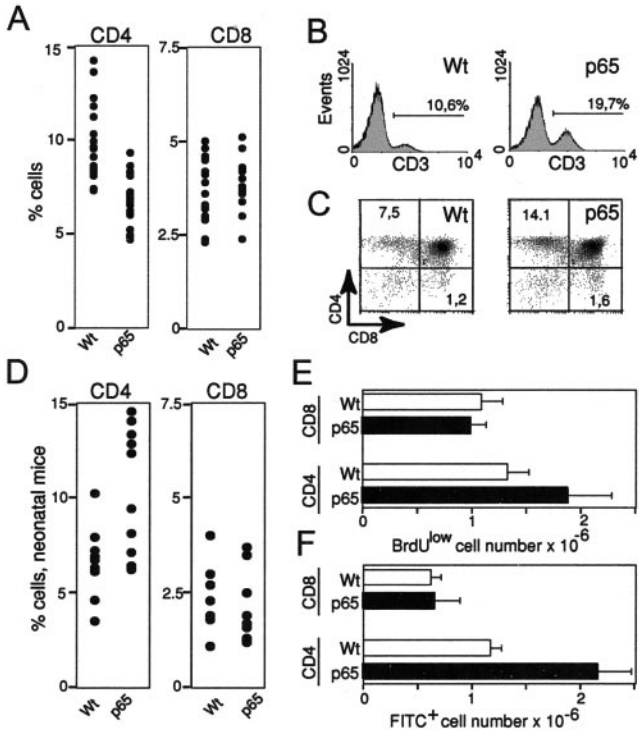

FIGURE 2. Enhanced class IA PI3K activation increases $CD4^+$ T cell generation. *A*, Thymocytes from 5- to 8-wk-old $p65^{PI3K}$ Tg or Wt littermates were stained with anti-CD4 and -CD8 mAbs, and the percentage of $CD4^+$ and $CD8^+$ cells was analyzed by flow cytometry. Each dot represents a single mouse. *B*, Thymocytes from neonatal $p65^{PI3K}$ Tg or Wt littermates were stained with anti-CD3 mAb and were analyzed by flow cytometry. The percentage of $CD3^{bright}$ cells is indicated. *C*, Thymocytes as in *B* were stained with anti-CD4 and -CD8 mAbs, and the percentage of $CD4^+$ and $CD8^+$ cells was analyzed by flow cytometry. *B* and *C*, Representative experiment. *D*, Thymocytes from neonatal $p65^{PI3K}$ Tg or Wt littermates were stained with anti-CD4 and -CD8 mAb, and the percentage of $CD4^+$ and $CD8^+$ cells was analyzed by flow cytometry. Each dot represents a single mouse. *E*, BrdU was administered for 8 days in drinking water to 5- to 8-wk-old Wt and $p65^{PI3K}$ Tg littermates, after which the presence of spleen T cells incorporating $BrdU^{low}$ was examined. The mean ± SD is representative of 12 mice from four litters examined. *F*, Thymocytes of 5- to 8-wk-old Wt and $p65^{PI3K}$ Tg littermates were FITC labeled in vivo by intrathymic injection. After 48 h, mice were sacrificed, and the appearance of FITC-labeled $CD4^+$ or $CD8^+$ in the spleen was quantitated. The mean ± SD is representative of 10 mice from three litters examined.

differences in the percentage of $CD8^+$ thymocytes (Fig. 2*A*). We also observed a decrease in the percentage of mature $CD3^{high}$ cells in Tg thymus (data not shown), indicating that the reduction in $CD4^+$ cells is not simply due to down-regulation of the CD4 receptor. The decrease in mature $CD4^+$ thymocytes in 5- to 8-wk-old $p65^{PI3K}$ Tg mice may be caused by a defect in $CD4^+$ cell differentiation or may reflect more efficient $CD4^+$ cell exit to periphery. To analyze T cell development independently of thymic emigration, we examined neonatal mice (2–2.5 days after birth), when thymocytes have just begun to populate the periphery (2). Neonatal $p65^{PI3K}$ Tg mice showed increased percentages of mature $CD3^{high}$ (Fig. 2*B*) and mature $CD4^+$ thymocytes (Fig. 2, *C* and *D*; $p < 0.001$). The distribution of CD44/CD25-based DN cell subpopulations was similar to that in 5- to 8-wk-old Tg mice (data not shown). The increase in the $CD4^+$ cell subpopulation was from a mean of 6.9 ± 1.6% in controls to 11.4 ± 2.8% in $p65^{PI3K}$ Tg mice. The proportion of thymic $CD4^+$ cells subsequently decreased and, between postnatal days 7 and 10, reached values similar to those of 1-mo-old Tg mice. These results indicate that $p65^{PI3K}$ increases the magnitude of positive selection, promoting generation of $CD4^+$ cells, as revealed in neonatal mice.

As the proportion of $CD4^+$ cells decreased in the days after birth, we postulated that $p65^{PI3K}$ expression could enhance $CD4^+$ thymocyte emigration. To analyze migration efficiency, we performed in vivo BrdU labeling, which results in $BrdU^{low}$ incorporation in most developing thymocytes (28, 32). The appearance of $BrdU^{low}$-labeled cells in the periphery allows quantitation of thymic migration (28). Although both types of mice incorporated BrdU similarly (60–70% of thymocytes labeled), the percentage of $BrdU^{low}$ $CD4^+$ cells in periphery was higher in $p65^{PI3K}$ Tg than in Wt mice (Fig. 2*E*; $p = 0.007$), indicating that $CD4^+$ thymocytes exit the thymus more efficiently in Tg mice. As an alternative approach, FITC was injected into the thymus of Wt and $p65^{PI3K}$ Tg mice; 48 h postinjection, we examined the appearance of FITC-labeled recent thymic emigrants in the periphery (29, 30, 33). The number of FITC-labeled $CD4^+$ thymic emigrants was consistently higher in $p65^{PI3K}$ Tg than in Wt littermates (Fig. 2*F*; $p = 0.006$). The $p65^{PI3K}$ transgene thus enhances generation of $CD4^+$ thymocytes and increases their exit to the periphery.

p110γ deficiency impairs DN cell differentiation

$p65^{PI3K}$ Tg expression affected DN cell differentiation and increased $CD4^+$ cell generation (Figs. 1 and 2). Because $p110\gamma^{-/-}$ mice have a smaller thymus and showed a decreased peripheral $CD4^+$ population (21), we asked whether the phenotype of these mice might also result from a defect in DN cell differentiation and $CD4^+$ cell generation. Total thymocyte numbers were reduced in $p110\gamma^{-/-}$ compared with $p110\gamma^{+/-}$ littermates (Fig. 3*A*; $p = 0.001$), yielding mean values of 60.2 ± 12.2 × 10^6 cells in $p110\gamma^{+/-}$ mice and 40.0 ± 9.7 × 10^6 cells in $p110\gamma^{-/-}$ mice. The $p65^{PI3K}$ expression in $p110\gamma^{-/-}$ mice did not restore thymus size (Fig. 3*A*; $p = 0.05$; mean, 43.5 ± 9.8 × 10^6 cells).

The $p110\gamma^{-/-}$ mouse thymuses showed an increased percentage of DN cells (Fig. 3*B*; $p = 0.04$). Because the $p110\gamma^{-/-}$ thymus is smaller, we calculated absolute cell numbers in these animals to evaluate the status of the different T cell populations more accurately. DN cell numbers were also increased in $p110\gamma^{-/-}$ compared with $p110\gamma^{+/-}$ mice (Fig. 3*B*; $p = 0.02$). Moreover, the proportion of DN cells at the $CD44^-CD25^+$ stage (DN III) was higher in $p110\gamma^{-/-}$ compared with $p110\gamma^{+/-}$ mice (Fig. 3*C*; $p = 0.001$), whereas the remaining populations were not significantly affected. This result suggests that $p110\gamma^{-/-}$ mice have a partial defect at DN differentiation stage III. In agreement with a role for p110γ at the pre-TCR checkpoint, $p110\gamma^{-/-}$ mice had decreased proportions of large $CD44^-CD25^+$ cells (Fig. 3*D*; $p = 0.002$), cells that emerge after pre-TCR ligation (34). Introduction of the $p65^{PI3K}$ transgene in $p110\gamma^{-/-}$ mice complemented the pre-TCR differentiation defect, in that it reduced accumulation of DN cells and of $CD44^-CD25^+$ cells (Fig. 3, *B* and *C*), increasing the appearance of large $CD44^-CD25^+$ cells (Fig. 3*D*).

The $p110\gamma^{-/-}$ mice showed reduced percentages (Fig. 3*E*; $p = 0.0005$) and absolute numbers of DP cells (Fig. 3*E*; mean values, 48.0 ± 11.0 × 10^6 and 30.9 ± 8.2 × 10^6 in $p110\gamma^{+/-}$ and $p110\gamma^{-/-}$ mice, respectively; $p = 0.008$). $p65^{PI3K}$ expression in $p110\gamma^{-/-}$ mice induced a moderate increase in the percentage of DP cells (Fig. 3*E*), but DP cell numbers and thymus size (Fig. 3, *A* and *E*) remained lower than in $p110\gamma^{+/-}$ mice. These observations suggest that $p110\gamma^{-/-}$ mice have a partial defect at the pre-TCR checkpoint of DN cell differentiation, which is complemented by $p65^{PI3K}$ transgene expression. Expansion of the DP pool nonetheless requires p110γ expression.

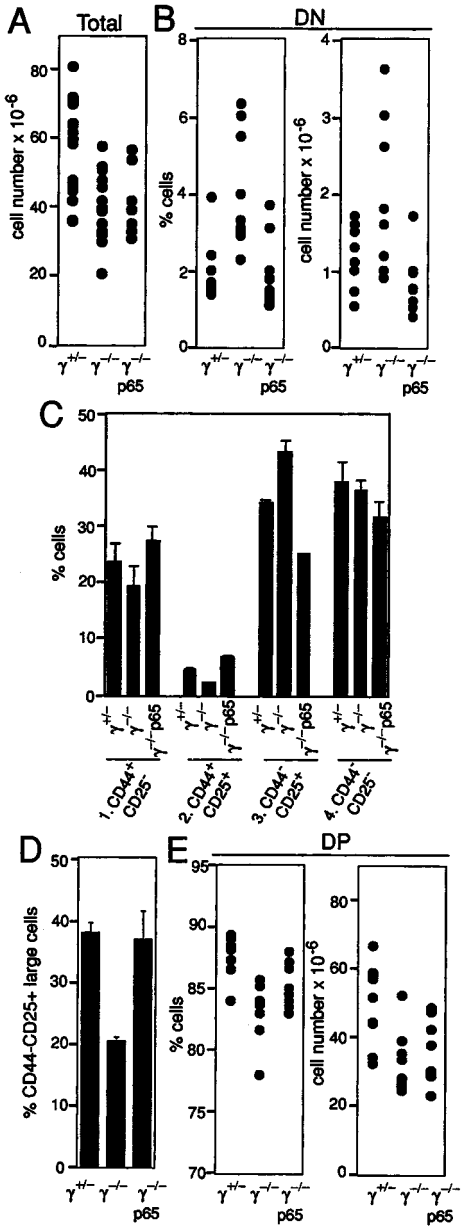

FIGURE 3. p110γ deficiency blocks DN cell differentiation. *A*, Thymocytes from 5- to 8-wk-old p110$\gamma^{+/-}$, p110$\gamma^{-/-}$, and p65^{PI3K} Tg/p110$\gamma^{-/-}$ littermates were isolated, and total cell number was counted for each thymus. Each dot represents a single mouse. *B*, Thymocytes as in *A* were isolated, counted, stained with anti-CD4 and -CD8 mAbs, and analyzed by flow cytometry. The figure shows the percentages and absolute numbers of DN cells. Each dot represents a single mouse. *C*, Thymocytes as in *A* were stained simultaneously with anti-CD4, -CD8, -CD44, and -CD25 mAbs. The CD4$^-$CD8$^-$ (DN) population was gated, and the percentage of the four indicated populations was examined. The mean ± SD is representative of ten p110$\gamma^{+/-}$ and p110$\gamma^{-/-}$ and seven p65^{PI3K} Tg/p110$\gamma^{-/-}$ mice. *D*, Cells were processed as in *C*. CD44$^-$CD25$^+$ cells were gated, and the proportion of large cells in this population was examined. *E*, Cells were processed as in *B*, and the percentage of DP cells was analyzed by flow cytometry.

p110γ deficiency decreases the CD4$^+$/CD8$^+$ differentiation ratio

p110γ regulates macrophage and neutrophil migration (25). To examine CD4$^+$ and CD8$^+$ cell differentiation independently of thymic emigration defects, we studied neonatal mice. No significant changes were detected in the percentage of CD4$^+$ cells (Fig. 4*A*), but the lower cellularity of p110$\gamma^{-/-}$ thymuses resulted in a moderate reduction in absolute CD4$^+$ cell numbers (Fig. 4*A*; $p = 0.005$). In contrast, the percentages and absolute numbers of CD8$^+$ cells were significantly increased in neonatal p110$\gamma^{-/-}$ compared with p110$\gamma^{+/-}$ mice (Fig. 4*B*; $p = 0.001$ and $p = 0.05$, respectively). As a consequence, p110$\gamma^{-/-}$ mice yielded a CD4$^+$/CD8$^+$ differentiation ratio near 1 (Fig. 4, *A* and *B*), rather than the normal CD4$^+$/CD8$^+$ cell differentiation ratio (more than 2-fold) observed in p110$\gamma^{+/-}$ mice. The p65^{PI3K} transgene expression in p110$\gamma^{-/-}$ mice increased generation of CD4$^+$ cells and decreased that of CD8$^+$ cells (data not shown). Fig. 4*C* shows a schematic representation of the proportion of CD4$^+$ and CD8$^+$ cells in the distinct

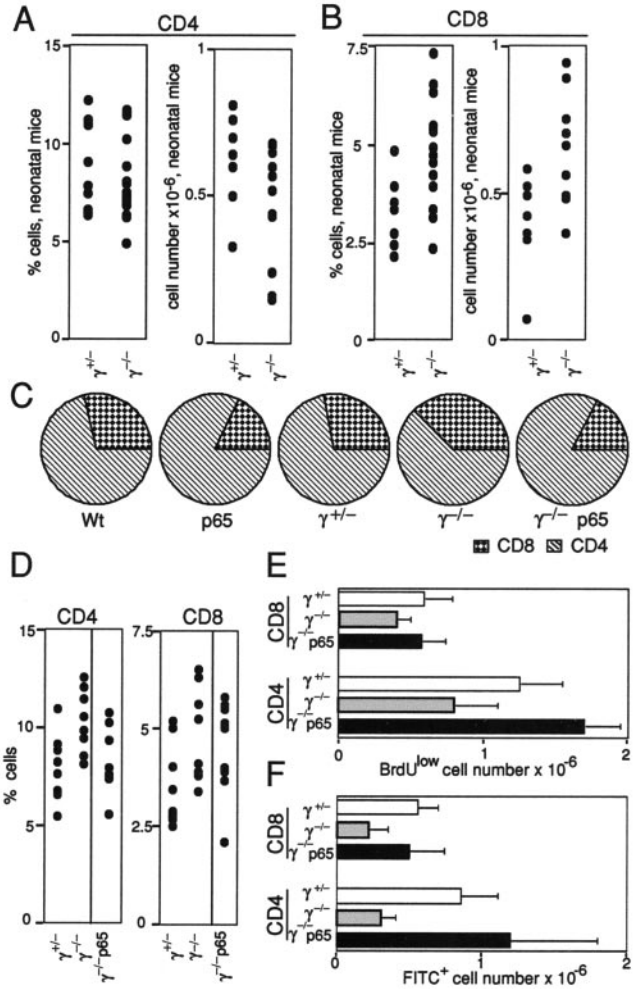

FIGURE 4. p110γ deficiency increases CD8$^+$ cell differentiation. *A* and *B*, Thymocytes from neonatal p110$\gamma^{+/-}$ and p110$\gamma^{-/-}$ littermates were isolated, counted, stained with anti-CD4 and -CD8 mAbs, and analyzed by flow cytometry. The figure illustrates the percentages and absolute numbers of mature CD4$^+$ (*A*) and CD8$^+$ (*B*) thymocytes. Each dot represents a single mouse. *C*, Thymocytes from neonatal Wt, p65^{PI3K} Tg, p110$\gamma^{+/-}$, p110$\gamma^{-/-}$, and p65^{PI3K} Tg/p110$\gamma^{-/-}$ mice were processed as in *A*. The mean proportion of differentiated CD4$^+$ and CD8$^+$ cells in each mouse is represented. Each analysis includes at least seven mice. *D*, Thymocytes from 5- to 8-wk-old p110$\gamma^{+/-}$, p110$\gamma^{-/-}$, and p65^{PI3K} Tg/p110$\gamma^{-/-}$ littermates were isolated, counted, stained with anti-CD4 and -CD8 mAbs, and analyzed by flow cytometry. The figure shows the percentages of CD4$^+$ and CD8$^+$ cells. Each dot represents a single mouse. *E*, CD4$^+$ or CD8$^+$ thymic emigrants from the different mice (indicated) were examined as in Fig. 2*E*. *F*, CD4$^+$ or CD8$^+$ thymic emigrants from the different mice (indicated) were examined as in Fig. 2*F*.

neonatal mice. Whereas $p65^{PI3K}$ expression enhanced $CD4^+$ cell differentiation, p110γ deficiency enhanced $CD8^+$ cell generation.

In contrast with neonatal mice, 5- to 8-wk-old $p110\gamma^{-/-}$ mice showed increased percentages of both $CD4^+$ and $CD8^+$ thymocytes compared with $p110\gamma^{+/-}$ mice (Fig. 4*D*; $p = 0.005$ and $p = 0.009$, respectively). We postulated that p110γ deficiency might impair mature thymocyte exit to the periphery, causing accumulation of mature thymic residents. We examined $CD4^+$ and $CD8^+$ thymocyte migration. Compared with $p110\gamma^{+/-}$ mice, a significant reduction was found in the proportion of $CD4^+$ and $CD8^+$ $BrdU^{low}$ thymic emigrants in the periphery of $p110\gamma^{-/-}$ mice (Fig. 4*E*; $p = 0.01$ and $p = 0.006$, respectively). We also examined thymocyte migration by intrathymic FITC injection. The proportion of FITC-labeled $CD4^+$ and $CD8^+$ recent thymic emigrants was lower in $p110\gamma^{-/-}$ than in $p110\gamma^{+/-}$ mice (Fig. 4*F*; $p = 0.006$ and $p = 0.004$, respectively). These results demonstrate p110γ involvement in the regulation of thymic emigration.

Introduction of the $p65^{PI3K}$ transgene in $p110\gamma^{-/-}$ mice nonetheless increased $CD4^+$ and $CD8^+$ cell migration (Fig. 4, *E* and *F*), leading to a reduction in the percentage of mature $CD4^+$ and $CD8^+$ thymocytes in 5- to 8-wk-old $p65^{PI3K}$ Tg/$p110\gamma^{-/-}$ mice (Fig. 4*D*). Examination of neonatal mice showed that p110γ deficiency decreased $CD4^+$ cell differentiation and enhanced $CD8^+$ cell generation, altering the $CD4^+/CD8^+$ cell differentiation ratio. In addition, p110γ regulated mature thymocyte migration to the periphery.

p110γ and class IA p85/p110 PI3K affect the $CD4^+/CD8^+$ cell differentiation ratio in TCR Tg mice

To confirm the role of PI3K in determining the $CD4^+/CD8^+$ outcome of positive selection, we crossed $p110\gamma^{-/-}$ mice and $p65^{PI3K}$ Tg with TCR Tg mice. The $p110\gamma^{-/-}$ mice were crossed with mice expressing an MHC class II-restricted Tg TCR (5CC7 Tg mice) (26) that triggers $CD4^+$ cell development in the appropriate MHC context. 5CC7/$p110\gamma^{+/-}$ ($IE^{k/b}$) mice were obtained and compared with 5CC7/$p110\gamma^{-/-}$ ($IE^{k/b}$) littermates. Differentiation was examined in the first week of life, to avoid interference with thymic emigration defects in $p110\gamma^{-/-}$ mice. Compared with 5CC7/$p110\gamma^{+/-}$ mice, 5CC7/$p110\gamma^{-/-}$ mice showed a significant decrease in the percentage of differentiated $CD4^+$ cells and a significant increase in the percentage of differentiated $CD8^+$ cells (Fig. 5*A*). This shift in the $CD4^+/CD8^+$ differentiation ratio was also detected within the subpopulation of thymocytes expressing high levels of transgenic TCR Vβ- and Vα-chains (Fig. 5*A*). This suggests that the decrease in the $CD4^+/CD8^+$ differentiation ratio occurs within the Tg TCR^+ cell population, although this MHC class II-restricted TCR normally induces $CD4^+$ cell differentiation. The increase in $CD8^+$ cell differentiation was also observed in the HSA-negative (HSA^-) subpopulation, which represents the most mature thymus cell population (12) (Fig. 5*B*). The increase in $CD8^+$ cells that express an MHC class II-restricted TCR and the concomitant decrease in transgenic TCR^+CD4^+ cell differentiation suggest that p110γ deficiency influences $CD4^+/CD8^+$ differentiation fate.

$CD4^+$ cell differentiation is increased in $p65^{PI3K}$ Tg mice (Fig. 2), indicating that enhancement of class IA PI3K activation may also modulate $CD4^+/CD8^+$ cell differentiation. To examine this possibility, $p65^{PI3K}$ Tg mice were crossed with F5 Tg mice expressing an MHC class I-restricted transgenic TCR (27) that drives $CD8^+$ thymocyte development in the appropriate MHC context. We observed that T cell differentiation begins at a slower rate in F5 Tg mice and that the proportion of $CD3^+$ mature thymocytes (mostly $CD8^+$ cells) continues to increase during the first week of life (data not shown). We thus examined 5- to 8-wk-old F5 Tg ($H\text{-}2^b$) and F5/$p65^{PI3K}$ double-Tg ($H\text{-}2^b$) mice. $p65^{PI3K}$ transgene expression in F5 mice reduced $CD8^+$ T cell differentiation (Fig. 6*A*). Because no appropriate Ab is available to examine expression of the transgenic Vα-chain (Vα4), in this case we only analyzed $CD4^+/CD8^+$ cell differentiation in the subpopulation of cells expressing the transgenic Vβ-chain. A decrease in $CD8^+$ cell differentiation was detected within the thymic population expressing high levels of the Tg TCR Vβ-chain (Fig. 6*A*).

Mature $CD4^+$ thymic residents were very low in both F5 Tg and F5/$p65^{PI3K}$ double-Tg mice (Fig. 6*A*). Nonetheless, an increase in $CD4^+$ cell differentiation was found systematically in the HSA^- population in F5/$p65^{PI3K}$ mice (Fig. 6*B*). $p65^{PI3K}$ expression increases $CD4^+$ thymic export (Fig. 2). Therefore, it was possible that $p65^{PI3K}$ expression induced Tg TCR^+CD4^+ cell exit to the periphery at a higher rate than in F5 Tg mice. In fact, a larger number of $CD4^+$ thymic emigrants was found in the periphery of 5- to 8-wk-old F5/$p65^{PI3K}$ mice (Fig. 6*C*). Moreover, consistent

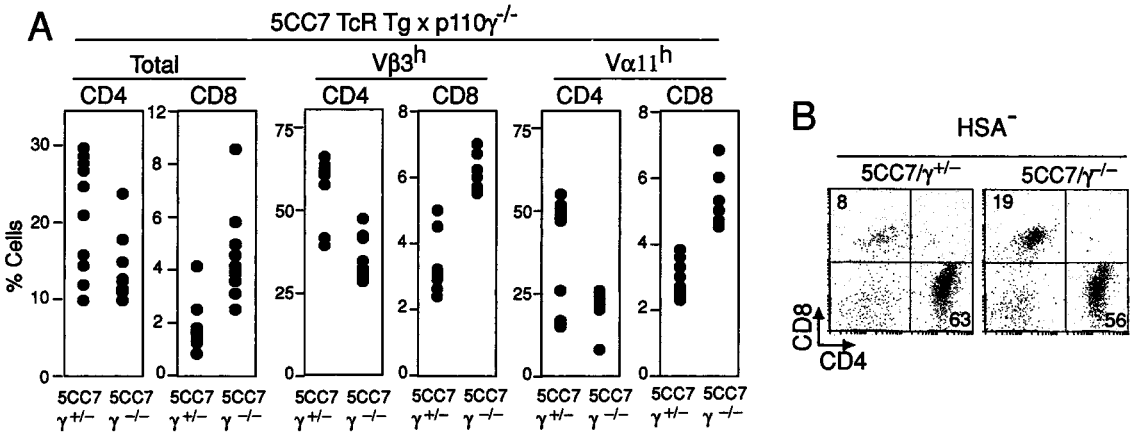

FIGURE 5. Class IB PI3K regulate $CD4^+/CD8^+$ cell fate. *A*, Thymocytes from 1-wk-old 5CC7/$p110\gamma^{+/-}$ and 5CC7/$p110\gamma^{-/-}$ littermates were isolated, stained with anti-CD4 and anti-CD8 mAbs, and analyzed by flow cytometry. Thymocytes were also stained with anti-Vβ3, -CD4, and -CD8 mAbs. The proportion of $CD4^+$ and $CD8^+$ cells within the population expressing high Vβ3 levels was examined. A similar analysis was performed of the $V\alpha11^{high}$ population (indicated). Each dot represents a single mouse. *B*, Thymocytes as in *A* were stained with anti-HSA, -CD4, and -CD8 mAbs, and then were analyzed by flow cytometry. The HSA^- population was gated, and the proportion of $CD4^+$ and $CD8^+$ cells in this population examined. The figure illustrates a representative mouse.

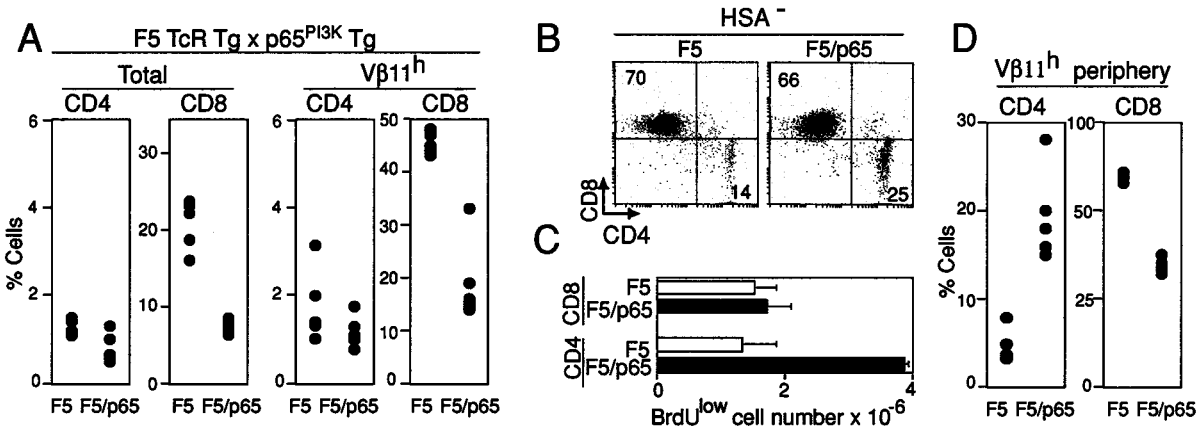

FIGURE 6. Class IA PI3K regulates $CD4^+/CD8^+$ cell fate. *A*, Thymocytes from 5- to 8-wk-old F5/$p65^{PI3K}$ Tg and F5 Tg littermates were isolated, stained with anti-CD4 and -CD8 mAbs, and analyzed by flow cytometry. Thymocytes were also stained with anti-Vβ11, -CD4, and -CD8 mAbs. The proportion of $CD4^+$ and $CD8^+$ cells within the population expressing $V\beta11^{high}$ was examined. Each dot represents a single mouse. *B*, Thymocytes as in *A* were stained with anti-HSA, -CD4, and -CD8 mAbs and were analyzed by flow cytometry. The HSA^- population was gated, and the proportion of $CD4^+$ and $CD8^+$ cells in this population was examined. The figure shows a representative mouse. *C*, $BrdU^{low}$ $CD4^+$ or $CD8^+$ thymic emigrants from the indicated mice (as in *A*) were examined as in Fig. 2*E*. *D*, Peripheral T cells from 5- to 8-wk-old F5/$p65^{PI3K}$ Tg and F5 Tg littermates were also stained with anti-Vβ11, -CD4, and -CD8 mAbs. The proportion of $CD4^+$ and $CD8^+$ cells within the population expressing $V\beta11^{high}$ was examined. Each dot represents a single mouse.

with the enhanced $CD4^+$ cell generation in F5/$p65^{PI3K}$ mice, peripheral $CD4^+$ cell numbers were increased in these animals compared with F5 Tg mice ($CD4^+$ splenocyte mean values, $4.6 \pm 1.7 \times 10^6$ and $10.7 \pm 3.1 \times 10^6$, respectively). Because Tg Vα-chains cannot be examined, we analyzed other Vα-chains (Vα3, Vα11, and Vα11.2); the expression levels of these Vα in F5 Tg mice were very low and were not significantly increased in F5/$p65^{PI3K}$ double-Tg mice (data not shown). Moreover, the proportion of $CD4^+$ cells expressing the transgenic TCR Vβ-chain in the periphery of F5/$p65^{PI3K}$ double-Tg mice was higher than in F5 Tg mice (Fig. 6*D*). This suggests that $p65^{PI3K}$ expression increases differentiation of $CD4^+$ cells that express the transgenic MHC class I-restricted TCR.

Discussion

We examined thymic development in p110γ-deficient mice and in mice expressing an activating mutation of class IA PI3K, $p65^{PI3K}$, in T cells. Both genetic alterations had a moderate effect on pre-TCR-induced DN cell differentiation; p110γ deficiency impaired this transition, and enhanced activation of class IA PI3K favored it. p110γ also regulated thymus growth. Nonetheless, the most striking observation in mice with genetic alterations in PI3K was the effect on $CD4^+/CD8^+$ lineage commitment. p110γ deficiency augmented $CD8^+$ cell differentiation, whereas enhancement of class IA PI3K activation increased generation of $CD4^+$ cells. These observations support the idea that the magnitude of PI3K activation regulates the $CD4^+/CD8^+$ T cell differentiation ratio. In addition, both alterations influenced migration of mature thymocytes to the periphery.

p110γ involvement in pre-TCR-dependent T cell differentiation is supported by the observation that $p110\gamma^{-/-}$ mice show an increase in DN cell numbers. These cells accumulated preferentially at DN stage III, the stage at which pre-TCR triggers differentiation (1, 2). The proportion of large cells at DN stage III, generated after pre-TCR ligation (34), is reduced by 50% in these mice. In support of a role for PI3K at this stage, $p65^{PI3K}$ mice showed reduced numbers of DN cells and a decreased percentage of DN stage III cells. $p65^{PI3K}$ expression complemented p110γ function, reducing DN cell accumulation at stage III and restoring the normal proportion of $CD44^-CD25^+$ large cells in $p110\gamma^{-/-}$ mice. These results show that class IA PI3K complements p110γ function. This complementation may reflect that both isoforms regulate this transition physiologically or that enhanced $p65^{PI3K}$-induced PI3K activity artificially compensates for the lack of p110γ. The $p65^{PI3K}$ expression nonetheless enhances basal 3-polyphosphoinositide levels only moderately and requires stimulation of a tyrosine kinase-coupled receptor to enhance PI3K activity significantly (22, 23). Pre-TCR, a receptor coupled to Lck tyrosine kinase (5, 35), thus may activate class IA PI3K isoforms physiologically, thereby contributing to pre-TCR-mediated differentiation, as observed in $p65^{PI3K}$ Tg mice. Moreover, the observation that $p65^{PI3K}$ expression does not restore thymus growth in $p110\gamma^{-/-}$ mice argues against a generalized compensation of p110γ function by increased 3-polyphosphoinositide levels in $p65^{PI3K}$ Tg mice. Nonetheless, whereas the phenotype of $p110\gamma^{-/-}$ mice demonstrates a function for p110γ in pre-TCR-dependent differentiation events, further studies in conditional class IA PI3K knockout mice are required to clarify the contribution of these isoforms.

The $p110\gamma^{-/-}$ mice show a defect in the proliferative expansion that accompanies development from the DN to the DP stage (21) (Fig. 4). p110γ is essential for this function, because enhanced class IA PI3K activation caused by $p65^{PI3K}$ expression did not restore normal thymus size in these mice. Thymus growth thus may require signals other than those needed for DN-to-DP differentiation. We postulate that a receptor that specifically regulates p110γ is involved in control of DP cell expansion. Pertussis toxin treatment resulted in a selective decrease in DP cells (36), supporting GPCR involvement in regulating DP cell expansion. Rho, a GPCR effector, also controls thymus growth (37, 38). Because both p110γ and Rho are activated by GPCR (39–41), receptors of this family are potential candidates for thymus growth regulation.

Mice with genetic modifications in PI3K showed altered $CD4^+/CD8^+$ differentiation ratios. The contribution of p110γ in the control of lineage commitment was evident in neonatal $p110\gamma^{-/-}$ mice, which display increased $CD8^+$ and decreased $CD4^+$ cell numbers. Crossing these animals with mice expressing an MHC class II-restricted Tg TCR, which normally induces $CD4^+$ cell

differentiation, resulted in an increase in $CD8^+$ cells and a reduction in $CD4^+$ cells expressing the Tg TCR. This demonstrates that p110γ activation is a component of the signaling pathways that regulate differentiation to $CD8^+$ or $CD4^+$ lineages. In the case of enhanced class IA PI3K activation, the result is consistent and complementary. In MHC class I-restricted TCR Tg mice that normally induce predominantly $CD8^+$ cell differentiation, $p65^{PI3K}$ expression increased generation of $CD4^+$ cells and reduced that of $CD8^+$ cells. In addition to this alteration in lineage commitment, $p65^{PI3K}$ expression mediated a quantitative increase in positive selection of $CD4^+$ cells. This is supported by the observation that neonatal $p65^{PI3K}$ mice have a larger number of mature $CD3^+$ T cells. We found no significant decrease in positive selection in $p110\gamma^{-/-}$ mice. Nonetheless, when $p110\gamma^{-/-}$ mice were crossed with 5CC7 TCR Tg mice, a decrease of ~25–30% was detected in the number of mature $CD3^+$ thymocytes. These data indicate that, in addition to the effect on $CD4^+/CD8^+$ lineage commitment, PI3K may also regulate the magnitude of positive selection.

To understand the mechanism by which enhanced PI3K activation increases positive selection of $CD4^+$ cells, we considered a reduction in apoptosis. Expression of the $p65^{PI3K}$ mutant (22) did not affect spontaneous apoptosis, γ- or UV-irradiation-induced apoptosis, or apoptosis induced by anti-Fas Ab, dexamethasone, staphylococcal enterotoxin B, or TNF-α (data not shown). We also examined Ag-induced apoptosis in $p65^{PI3K}$ Tg mice crossed with the 5CC7 TCR Tg mice (26). Administration of the specific antigenic peptide resulted in a comparable reduction in DP cells in 5CC7 and 5CC7 $\times$ $p65^{PI3K}$ Tg mice (data not shown). Thus, we find no reduction in apoptosis in $p65^{PI3K}$ Tg mice. In addition to increased tumor susceptibility, mice lacking Pten, a phosphatase that reduces 3-polyphosphoinositide levels generated by any PI3K isoform, showed decreased negative selection and increased generation of $CD4^+$ T cells with an activated phenotype (42). Thus, it is possible that strong induction of the PI3K pathway, such as that originated by Pten deletion, is required to affect negative selection and quantitatively increase positive selection. Even in the case of Pten, the authors report a selective increase in $CD4^+$ cells (42). This and the increase in peripheral $CD4^+$ T cells in other mouse models showing PI3K protein kinase B (PKB) pathway activation (43, 44) supports the conclusion that this route skews differentiation toward cells of the $CD4^+$ phenotype. Conversely, $p110\gamma^{-/-}$ mice have a reduced peripheral $CD4^+$ population (21).

It was recently proposed that the difference between signals that generate $CD4^+$ and $CD8^+$ cell differentiation is quantitative (12, 13). Enhanced activation of Lck or MAPK pathways promotes $CD4^+$ cell differentiation, whereas defective induction of these kinases increases $CD8^+$ cell differentiation (12, 13). PI3K acts according to this model, because p110γ deletion promotes $CD8^+$ generation (even when these cells express an MHC class II-restricted TCR), and transient enhanced class IA PI3K activation promotes $CD4^+$ generation (even when these cells express an MHC class I-restricted TCR). The magnitude of this shift is similar to that observed when there are genetic alterations in the MAPK pathway (13), and it is lower than those observed when Lck activity is altered (12). This may reflect the hierarchy of the signaling pathways that regulate differentiation. Lck is a Tyr kinase that is induced shortly after TCR ligation (35). Tyrosine kinases activate PI3K and MAPK (14, 23, 25, 45); thus, these two pathways may cooperate to control the $CD4^+/CD8^+$ cell differentiation ratio. In support of this view, MAPK and Lck control pre-TCR-mediated differentiation and CD4/CD8 lineage commitment. Nonetheless, $Lck^{-/-}$ mice have a more severe T cell differentiation defect (5, 12) than do $MAPK^{-/-}$ mice (8, 13, 46).

Class IA PI3K is triggered by TCR ligation (17). p110γ involvement in pre-TCR and TCR-regulated differentiation events was nonetheless unexpected, because these receptors trigger Tyr kinase activation (35), and p110γ is normally activated by GPCR (14). This suggests either that a GPCR cooperates with the pre-TCR and TCR to trigger p110γ activation, or that the pre-TCR and TCR activate p110γ. We have compared TCR-triggered PKB activation in thymocytes and T cells obtained from Wt, $p65^{PI3K}$ Tg, and $p110\gamma^{-/-}$ mice. PKB activation shortly after TCR binding was lower in $p110\gamma^{-/-}$ than in Wt mouse cells, and it was higher in cells from $p65^{PI3K}$ Tg or $p110\gamma^{-/-}/p65^{PI3K}$ Tg mice (data not shown). These results suggest that p110γ participates in TCR-triggered early signals, which may aid in elucidating the way in which this PI3K isoform regulates TCR-mediated differentiation. Future experiments will be oriented to define the mechanism by which TCR activates p110γ.

In addition to these effects on thymic differentiation, lack of p110γ reduces migration of mature thymocytes to the periphery. Moreover, the enhanced class IA PI3K activation by $p65^{PI3K}$ promotes $CD4^+$ cell migration and reconstitutes mature $CD4^+$ and $CD8^+$ T cell migration in $p110\gamma^{-/-}$ mice. This suggests that class IA and IB PI3K may both regulate thymocyte migration to the periphery. Because some receptors that trigger cell migration, such as chemokine receptors, induce class IB PI3K activation and subsequent IA PI3K stimulation (47–49), activation of these two enzymes in cascade may regulate thymocyte emigration.

The results illustrate that PI3K modulates T cell differentiation at several stages. The p110γ isoform regulates thymic growth. In addition, p110γ influences the pre-TCR-dependent DN-to-DP cell transition and affects the CD4/CD8 T cell differentiation ratio. Class IA p85/p110 PI3K-enhanced activation affects the same differentiation steps except for thymus growth, suggesting that class IA PI3K may contribute to regulation of T cell development. The observations presented demonstrate for the first time the contribution of p110γ to the signaling pathways that regulate pre-TCR-dependent differentiation and $CD4^+/CD8^+$ lineage commitment during positive selection.

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