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

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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, p65PI3K, 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 p65PI3K activating mutation. Genetic alteration of both PI3K isoforms also affects positive selection; p110γ deletion decreased and p65PI3K 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.

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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, p65PI3K, in T cells. This mutant regulatory subunit associates with the p110 catalytic subunit, moderately increasing basal PI-3,4,5-P3 levels and significantly enhancing the transient PI3K activation that follows receptor activation (22, 23). p65PI3K 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, p65PI3K 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 p65PI3K expression augmented this ratio. Moreover, p65PI3K expression corrected p110γ mouse differentiation defects, except for thymus growth. These results illustrate that PI3K regulates the thymic development of pre-TCR-dependent DN-to-DP 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**

p65PI3K 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 (Vβ3/Vα11) and F5 TCR (Vβ11/Vα8) 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 p65PI3K 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 were 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 Ab (HSA, CD24, M1/69), CD8 (L3T4, H129.19), CD3 (145-2C11), CD25 (IL-2R-α chain), CD44 (gpi), 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). TCRαβ Ab (H57-S97) 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 administered for 8 days in drinking water to wild type (Wt) and p65PI3K Tg mice, and then the presence of spleen T cells incorporating BrdUlow was examined by flow cytometry. Intrathymic FITC injections and analysis of recent thymic emigrants were as described (29, 30).

**Results**

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

We previously reported that young mice expressing the p65PI3K 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, p65PI3K 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 p65PI3K may affect thymic development. Here we examine T cell differentiation in young (5- to 8-wk-old) p65PI3K 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. 1A; Student’s t test; p = 0.0001). This represented a systematic reduction in absolute DN cell numbers from a mean of 7.4 ± 2.9 × 106 (Wt thymus) to 4.4 ± 2.2 × 106 (Tg thymus). Examination of DN differentiation steps (31) showed a significant decrease in the percentage of CD44+CD25+ cells (DN stage III) in p65PI3K Tg compared with Wt mice (Fig. 1B; p = 0.002) and no significant changes in the remaining populations. The percentage of DP cells was moderately increased in Tg mice (Fig. 1C; p = 0.0008). The p65PI3K 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.

**p65PI3K increases CD4+ T cell generation**

We next examined mature thymic T cell populations. The p65PI3K Tg mouse thymuses had lower percentages of CD4+ cells compared with Wt animals (Fig. 2A; p = 0.007), with no significant

![FIGURE 1.](http://www.jimmunol.org/) Enhanced class IA PI3K activation increases DN cell differentiation. A, Thymocytes from 5- to 8-wk-old p65PI3K 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 p65PI3K 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 ± SE from 12 mice examined is represented. C, Cells were processed as in A, and the percentage of DP cells was analyzed by flow cytometry.
CD4/H11006 generation. 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 p65PI3K 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 p65PI3K 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 p65PI3K Tg than in Wt mice (Fig. 2E; 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 p65PI3K 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 p65PI3K Tg than in Wt littermates (Fig. 2F; p = 0.006).

The p65PI3K transgene thus enhances generation of CD4+ thymocytes and increases their exit to the periphery.

**FIGURE 2.** Enhanced class IA PI3K activation increases CD4+ T cell generation. A. Thymocytes from 5- to 8-wk-old p65PI3K 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 p65PI3K Tg or Wt littermates were stained with anti-CD3 mAb and were analyzed by flow cytometry. The mean ± SD is representative of 12 mice from four litters examined. C. Thymocytes from 5- to 8-wk-old Wt and p65PI3K 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+ cells in the spleen was quantitated. The mean ± SD is representative of 10 mice from three litters examined.

**p110y deficiency impairs DN cell differentiation.** p65PI3K Tg expression affected DN cell differentiation and increased CD4+ cell generation (Figs. 1 and 2). Because p110y+/- 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 p110y+/- compared with p110y+/- littermates (Fig. 3A; p = 0.001), yielding mean values of 60.2 ± 12.2 × 10^6 cells in p110y+/- mice and 40.0 ± 9.7 × 10^6 cells in p110y+/- mice. The p65PI3K expression in p110y+/- mice did not restore thymus size (Fig. 3A; p = 0.05; mean, 43.5 ± 9.8 × 10^6 cells).

The p110y+/- mouse thymuses showed an increased percentage of DN cells (Fig. 3B; p = 0.04). Because the p110y+/- 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 p110y+/- compared with p110y+/- mice (Fig. 3B; p = 0.02). Moreover, the proportion of DN cells at the CD44+CD25+ stage (DN III) was higher in p110y+/- compared with p110y+/- mice (Fig. 3C; p = 0.001), whereas the remaining populations were not significantly affected. This result suggests that p110y+/- mice have a partial defect at DN differentiation stage III. In agreement with a role for p110y at the pre-TCR checkpoint, p110y+/- mice had decreased proportions of large CD44+CD25+ cells (Fig. 3D; p = 0.002), cells that emerge after pre-TCR ligation (34). Introduction of the p65PI3K transgene in p110y+/- 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. 3D).

The p110y+/- mice showed reduced percentages (Fig. 3E; p = 0.0005) and absolute numbers of DP cells (Fig. 3E; mean values, 48.0 ± 11.0 × 10^6 and 30.9 ± 8.2 × 10^6 in p110y+/- and p110y+/- mice, respectively; p = 0.008). p65PI3K expression in p110y+/- mice induced a moderate increase in the percentage of DP cells (Fig. 3E), but DP cell numbers and thymus size (Fig. 3, A and E) remained lower than in p110y+/- mice. These observations suggest that p110y+/- mice have a partial defect at the pre-TCR checkpoint of DN cell differentiation, which is complemented by p65PI3K transgene expression. Expansion of the DP pool nonetheless requires p110y expression.

differences in the percentage of CD8+ thymocytes (Fig. 2A). We also observed a decrease in the percentage of mature CD3high 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 p65PI3K 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 p65PI3K Tg mice showed increased percentages of mature CD3high (Fig. 2B) 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 p65PI3K 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 p65PI3K 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 p65PI3K 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 p65PI3K Tg than in Wt mice (Fig. 2E; 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 p65PI3K 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 p65PI3K Tg than in Wt littermates (Fig. 2F; p = 0.006). The p65PI3K transgene thus enhances generation of CD4+ thymocytes and increases their exit to the periphery.
The CD4 stained simultaneously with anti-CD4, -CD8, -CD44, and -CD25 mAbs. Each dot represents a single mouse. Flow cytometry. The percentage and absolute numbers of CD4 or CD8 cells were significantly increased in neonatal p110γ−/− compared with p110γ+/+ mice (Fig. 4B; p = 0.001 and p = 0.05, respectively). As a consequence, p110γ−/− mice yielded a CD4+/CD8+ differentiation ratio near 1 (Fig. 4A and B), rather than the normal CD4+/CD8+ cell differentiation ratio (more than 2-fold) observed in p110γ+/+ mice. The p65PI3K transgene expression in p110γ−/− mice increased generation of CD4+ cells and decreased that of CD8+ cells (data not shown). Fig. 4C shows a schematic representation of the proportion of CD4+ and CD8+ cells in the distinct thymic emigrants from the different mice.

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. 4A), but the lower cellularity of p110γ−/− thymuses resulted in a moderate reduction in absolute CD4+ cell numbers (Fig. 4A; p = 0.005). In contrast, the percentages and absolute numbers of CD8+ cells were significantly increased in neonatal p110γ−/− compared with p110γ+/+ mice (Fig. 4B; p = 0.001 and p = 0.05, respectively). As a consequence, p110γ−/− mice yielded a CD4+/CD8+ differentiation ratio near 1 (Fig. 4A and B), rather than the normal CD4+/CD8+ cell differentiation ratio (more than 2-fold) observed in p110γ+/+ mice. The p65PI3K transgene expression in p110γ−/− mice increased generation of CD4+ cells and decreased that of CD8+ cells (data not shown). Fig. 4C shows a schematic representation of the proportion of CD4+ and CD8+ cells in the distinct thymic emigrants from the different mice.

FIGURE 3. p110γ deficiency blocks DN cell differentiation. A, Thymocytes from 5- to 8-wk-old p110γ−/−, p110γ−/−, and p65PI3K Tg/p110γ−/− littermates were isolated, and total cell number was counted for each thymus. Each dot represents a single mouse. B, Thymocytes as in A were stained simultaneously 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 p110γ−/− and p110γ−/− and seven p65PI3K Tg/p110γ−/− mice. D, Cells were processed as in C. CD4+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.

FIGURE 4. p110γ deficiency increases CD8+ cell differentiation. A and B, Thymocytes from neonatal p110γ−/− and p110γ−/− 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, p65PI3K Tg, p110γ−/−, p110γ−/−, and p65PI3K Tg/p110γ−/− 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 p110γ−/−, p110γ−/−, and p65PI3K Tg/p110γ−/− 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. 2E. F, CD4+ or CD8+ thymic emigrants from the different mice (indicated) were examined as in Fig. 2F.
neonatal mice. Whereas p65PI3K expression enhanced CD4+ cell differentiation, p110y deficiency enhanced CD8+ cell generation.

In contrast with neonatal mice, 5- to 8-wk-old p110y−/− mice showed increased percentages of both CD4+ and CD8+ thymocytes compared with p110y+/− mice (Fig. 4D; p = 0.005 and p = 0.009, respectively). We postulated that p110y deficiency might impair mature thymocyte exit to the periphery, causing accumulation of mature thymic residents. We examined CD4+ and CD8+ thymocyte migration. Compared with p110y+/− mice, a significant reduction was found in the proportion of CD4+ and CD8+ BrdUlow thymic emigrants in the periphery of p110y−/− mice (Fig. 4E; 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 p110y−/− than in p110y+/− mice (Fig. 4F; p = 0.006 and p = 0.004, respectively). These results demonstrate p110y involvement in the regulation of thymic emigration.

Introduction of the p65PI3K transgene in p110y−/− 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 p65PI3K Tg/p110y−/− mice (Fig. 4D). Examination of neonatal mice showed that p110y deficiency decreased CD4+ cell differentiation and enhanced CD8+ cell generation, altering the CD4+/CD8+ cell differentiation ratio. In addition, p110y regulated mature thymocyte migration to the periphery.

p110y 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 p110y−/− mice and p65PI3K Tg with TCR Tg mice. The p110y−/− mice were crossed with expressing a MHC class II-restricted Tg TCR (5CC7 Tg mice) (26) that triggers CD4+ cell development in the appropriate MHC context. 5CC7/p110y−/− (IE4kb) mice were obtained and compared with 5CC7/p110y+/− (IE4kb) littermates. Differentiation was examined in the first week of life, to avoid influence with thymic emigration defects in p110y−/− mice. Compared with 5CC7/p110y+/− mice, 5CC7/p110y−/− mice showed a significant decrease in the percentage of differentiated CD4+ cells and a significant increase in the percentage of differentiated CD8+ cells (Fig. 5A). 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. 5A). 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. 5B). 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 p110y deficiency influences CD4+/CD8+ differentiation fate.

CD4+ cell differentiation is increased in p65PI3K Tg mice (Fig. 2), indicating that enhancement of class IA PI3K activation may also modulate CD4+/CD8+ cell differentiation. To examine this possibility, p65PI3K Tg mice were crossed with F5 Tg mice expressing an MHC class II-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 examined 5- to 8-wk-old F5 Tg (H-2b) and F5/p65PI3K double-Tg (H-2b) mice. p65PI3K transgene expression in F5 mice reduced CD8+ T cell differentiation (Fig. 6A). 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. 6A).

Mature CD4+ thymic residents were very low in both F5 Tg and F5/p65PI3K double-Tg mice (Fig. 6A). Nonetheless, an increase in CD4+ cell differentiation was found systematically in the HSA− population in F5/p65PI3K mice (Fig. 6B). p65PI3K expression increases CD4+ thymic export (Fig. 2). Therefore, it was possible that p65PI3K 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/p65PI3K mice (Fig. 6C). Moreover, consistent

FIGURE 5. Class IB PI3K regulate CD4+/CD8+ cell fate. A, Thymocytes from 1-wk-old 5CC7/p110y−/− and 5CC7/p110y−/− 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 Va11h 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.
with the enhanced CD4+ cell generation in F5/p65PI3K mice, peripher-ral CD4+ cell numbers were increased in these animals compared with F5 Tg mice (CD4+ splenocyte mean values, 4.6 ± 1.7 × 10⁶ and 10.7 ± 3.1 × 10⁶, 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/p65PI3K double-Tg mice (data not shown). Moreover, the proportion of CD4+ cells expressing the transgenic TCR Vβ-chain in the periphery of F5/p65PI3K double-Tg mice was higher than in F5 Tg mice (Fig. 6D). This suggests that p65PI3K 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, p65PI3K, 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 p110γ−/− 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, p65PI3K mice showed reduced numbers of DN cells and a decreased percentage of DN stage III cells. p65PI3K expression complemented p110γ function, reducing DN cell accumulation at stage III and restoring the normal proportion of CD44+CD25+ large cells in p110γ−/− 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 p65PI3K-induced PI3K activity artificially compensates for the lack of p110γ. The p65PI3K expression nonetheless enhances basal 3-phosphoinositide 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 p65PI3K Tg mice. Moreover, the observation that p65PI3K expression does not restore thymus growth in p110γ−/− mice argues against a generalized compensation of p110γ function by increased 3-phosphoinositide levels in p65PI3K Tg mice. Nonetheless, whereas the phenotype of p110γ−/− 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 p110γ−/− 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 p65PI3K 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 p110γ−/− 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...
differential selection, which increases positive selection of CD4+ T cells. This indicates that p110γ activation is a component of the signaling pathways that promote 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, p65PI3K expression increased generation of CD4+ cells and reduced that of CD8+ cells. In addition to this alteration in lineage commitment, p65PI3K expression mediated a quantitative increase in positive selection of CD4+ cells. This is supported by the observation that neonatal p65PI3K mice have a larger number of mature CD3+ T cells. We found no significant decrease in positive selection in p110γ−/− mice. Nonetheless, when p110γ−/− mice were crossed with SCC7 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 p65PI3K mutant (22) did not affect spontaneous apoptosis, γ- and 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 p65PI3K Tg mice crossed with the SCC7 TCR Tg mice (26). Administration of the specific antigenic peptide resulted in a comparable reduction in DP cells in SCC7 and SCC7 × p65PI3K Tg mice (data not shown). Thus, we find no reduction in apoptosis in p65PI3K Tg mice. In addition to increased tumor susceptibility, mice lacking Pten, a phosphatase that reduces 3-phosphoinositide 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, p110γ−/− 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, p65PI3K Tg, and p110γ−/− mice. PKB activation shortly after TCR binding was lower in p110γ−/− than in Wt mouse cells, and it was higher in cells from p65PI3K Tg or p110γ−/−/p65PI3K 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 p65PI3K promotes CD4+ cell migration and reconstitutes mature CD4+ and CD8+ T cell migration in p110γ−/− 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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