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Phosphatidylinositol 3-Kinase Regulates Thymic Exit

Susannah D. Barbee and Jose Alberola-Ila

To understand the role of PI3K during T cell development, we generated transgenic mice expressing the N terminus of the PI3K catalytic subunit (p110<sub>ABD</sub>; ABD, adaptor binding domain) in thymocytes. Expression of p110<sub>ABD</sub> activates endogenous p110 and results in the accumulation of mature single-positive CD3<sup>hi</sup> Heat-stable Ag<sup>low</sup> thymocytes. This is mostly due to a defect in emigration of those cells, as shown by the delayed appearance of peripheral T cells in neonatal transgenic mice and by competitive adoptive transfer experiments. Although the mechanisms underlying these effects of PI3K are not yet clear, our results show an important role for PI3K activity in the regulation of mature thymocyte exit to the periphery. The Journal of Immunology, 2005, 174: 1230–1238.

During T cell development, the organism generates a T cell population with an extended repertoire of Ag specificities, but lacking autoreactivity. After the molding of this repertoire via signals derived from the interaction of TCR on thymocytes with their ligands, MHC molecules with bound peptides, thymocytes undergo a final maturation process before exiting to the periphery. Aside from gross phenotypic alterations, such as the down-regulation of CD69 and heat-stable Ag (HSA)<sup>3</sup> and the up-regulation of L-selectin (CD62L) surface expression, very little is understood about the final events of thymocyte development. In particular, the signals that regulate emigration of fully mature T cells to peripheral lymphoid tissues remain unknown. Mechanisms to explain thymocyte exit have mostly fallen into two models of active movement: chemorepulsion (or fugetaxis) from thymic stromal-derived elements or chemotraction to peripheral signals. A third alternative of passive regulation, the loss of responsiveness to thymic retention signals, has elicited less discussion.

Potential molecular candidates in these models have focused on G protein-coupled receptors because of the block in thymocyte emigration induced by transgenic expression of pertussis toxin (PTX), a G<sub>a</sub>-specific inhibitor, in thymocytes (1). The molecular pathways downstream of G protein-coupled receptors are poorly understood. These pathways can be broadly grouped into two categories: PTX-sensitive pathways downstream of the G<sub>a</sub> subunit, and PTX-insensitive pathways downstream of the G<sub>βγ</sub> heterodimer. One of the classic molecules activated by G<sub>α/βγ</sub> subunits is the class I<sub>α</sub> PI3K p110<sub>α</sub>. However, roles downstream of chemokine receptors have also been suggested for the class I<sub>α</sub> PI3K family members (reviewed in Ref. 2).

Class I<sub>α</sub> PI3Ks are activated by a variety of extracellular stimuli, including Ag receptor binding, and have been implicated in a wide range of cellular processes, such as cell cycle progression, cell growth, and cell survival (3–5). The class I<sub>α</sub> PI3Ks comprise a p110 catalytic subunit and a regulatory adapter subunit. Three isoforms of the p110 subunit have been described (α, β, and δ), and two mammalian genes (α and β) encode multiple adapter subunit isoforms. The prototypical model for PI3K activation involves recruitment of the p110 catalytic subunit to the membrane by the adapter subunit, which binds to phosphorylated tyrosines through its Src homology 2 domain (6). In the plasma membrane, p110 phosphorylates its main substrate phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)<sub>P</sub>) and thereby generates PtdIns(3,4,5)<sub>P</sub><sub>3</sub> and PtdIns(3,4)<sub>P</sub><sub>2</sub>, then recruits a number of PI3K effectors to the membrane by binding to their pleckstrin homology (PH) domains. The best-characterized PI3K effectors are Akt, the Rac exchange factor Vav-1, and some members of the Tec family of kinases. Akt contributes to the regulation of cell survival through multiple effectors, Vav-1 and Rac are involved in cytoskeletal reorganization, and the Tec kinases contribute to the activation of phospholipase C (PLC) and therefore to regulation of the intracellular calcium concentration (reviewed in Ref. 7).

Loss of PI3K in mice results in a profound block at the pro-B to pre-B transition that resembles the phenotype observed in xid mice, Btk<sup>−/−</sup> mice, and the human disease XL-linked gammaglobulinemia (8). However, no disruption of T lymphocyte development has been reported in a variety of PI3K knockouts, including all α adapter isoforms (p85α<sub>55α</sub>α<sub>50α</sub>α<sub>−/−</sub>) (9, 10) and p110<sub>δ</sub> (11). T cell development probably proceeds normally in these knockout mice due to redundancy, as suggested by the residual PI3K activity observed in T cells. Expression of a dominant negative form of p85<sup>Δp85</sup> in fetal thymic organ culture inhibited IL-7R-mediated proliferation and/or survival of thymocyte precursors without altering thymocyte differentiation, as assessed by the percentages of double-negative (DN), double-positive (DP), and single-positive (SP) thymocytes (12). In contrast, effects on the CD4/CD8 differentiation ratio have been reported in neonate p110<sub>δ</sub>−/− animals and transgenic mice expressing the mildly activating class I<sub>α</sub> p65<sup>PI3KΔ</sup> transgene (13).

Abbreviations used in this paper: HSA, heat-stable Ag; ABD, adaptor binding domain; DN, double negative (CD4<sup>−</sup>CD8<sup>−</sup>); DP, double positive (CD4<sup>+</sup>CD8<sup>+</sup>); NLC, nontransgenic littermate control; PH, pleckstrin homology; PLC, phospholipase C; PtdIns(4,5)<sub>P</sub><sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)<sub>P</sub><sub>3</sub>, PtdIns(3,4,5)<sub>P</sub><sub>3</sub>, PtdIns(3,4)<sub>P</sub><sub>2</sub>, PTX, pertussis toxin; SP, single positive; SIP<sub>α</sub>, sphingosine-1-phosphate; WT, wild type.

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p65\(^{\text{PI3K}}\) transgene and that CD4 and CD8 thymocyte exits are inhibited by p110\(_{\text{ty}}\) deficiency (13). We thus sought to further delineate the role of class I\(_{\text{A}}\) PI3K in thymocyte egress.

To understand the role of PI3K during T cell development, we have generated several lines of transgenic mice, referred to as p110\(_{\text{ABD}}\) (ABD, adaptor binding domain), that overexpress an N-terminal fragment of p110\(_{\alpha}\), containing the p85 adaptor-binding domain, in thymocytes. This construct induced the constitutive activation of PI3K, as measured by the activity of Akt, and its expression in the thymus yielded increased numbers of the more mature thymic subpopulations, CD3\(^{\text{high}}\)HSAlow SP thymocytes. p110\(_{\text{ABD}}\) markedly delayed the appearance of mature peripheral T cells in competitive adoptive transfer recipients as well as in neonatal mice.

Materials and Methods

Generation of p110\(_{\text{ABD}}\) transgenic mice

The p110\(_{\text{ABD}}\) construct, consisting of aa 1–108 of bovine class IA catalytic subunit of PI3K, was inserted into the embryonic stem (ES) cell line, WISH/2 (14), and the 9E10 c-Myc epitope was included at the N terminus to exist as an unstable monomer (Fig. 1B). Monomeric p110 subunits have constitutive lipid kinase activity, and indeed, we observed the constitutive activation of Akt, a well-known downstream effector of PI3K, in thymocytes of mice expressing the p110\(_{\text{ABD}}\) transgene (18). As shown in Fig. 1E by an in vitro kinase assay of Akt immunoprecipitates, unstimulated p110\(_{\text{ABD}}\) thymocytes displayed the same maximal level of Akt activity that was induced in nontransgenic thymocytes after 2 min of stimulation with plate-bound anti-CD3 Ab.

PI3K also activates Akt-independent pathways downstream of Ag receptors. PH domain-containing Tec kinases (and Itk, in particular) have been shown to regulate Ca\(^{2+}\) mobilization via PLC\(\gamma\) and its lipid products (19, 20). The control of PLC\(\gamma\) is complex (reviewed in Refs. 21 and 22), but PI3K has been described as a positive regulator (23). Therefore, we examined whether p110\(_{\text{ABD}}\) influenced calcium mobilization after TCR ligation. We used the Ca\(^{2+}\)-binding intracellular dye Indo-I in conjunction with fluorochrome-conjugated Abs to CD4 and CD8 to monitor changes in the intracellular calcium concentration in DP thymocytes from nontransgenic littermate controls (NLC) and p110\(_{\text{ABD}}\) mice in response to CD3 cross-linking at different concentrations. Fig. 1F shows the results of two representative experiments. The expression of p110\(_{\text{ABD}}\) in DP thymocytes strongly potentiated their Ca\(^{2+}\) flux responses to low doses of anti-CD3 mAb without modifying the maximal response. Thus, constitutive activation of PI3K improved the ability of DP to flux calcium in response to low intensity stimuli, allowing maximal Ca\(^{2+}\) flux responses to suboptimal anti-CD3 Ab concentrations. In contrast, other signal transduction pathways downstream from the TCR, such as the Ras/MAPK cascade, were not affected by expression of the p110\(_{\text{ABD}}\) transgene, suggesting that its effect is specific for PI3K-regulated pathways.

Flow cytometric analyses

For Ca\(^{2+}\) flux analyses, primary thymocytes (10⁶/ml) were labeled with Indo-1 (2 μg/ml in HBSS and 1% FBS; Molecular Probes) for 30 min at 37°C. Cells were centrifuged and then stained with azide-free anti-CD8-FITC and anti-CD4-PE for 20 min at room temperature. Samples were then diluted to 5 × 10⁵ cells/ml and incubated for ≥5 min at 37°C before analysis. Anti-CD3 Ab was added after 30 s, and goat anti-hamster Ab was added at 5 min. Maximal Ca\(^{2+}\) flux was measured after stimulation with 1–2 μg/ml ionomycin, and calibrations were performed with Calcium Calibration Buffer Kit 1 (Molecular Probes). The pre- and poststimulation Ca\(^{2+}\) levels corresponded to those previously reported as determined by comparison with a titration standard (data not shown) (15).

All Abs used were obtained from BD Pharmingen, and analyses were performed using a FACScalibur (BD Biosciences), with the exception of Ca\(^{2+}\) flux studies, which were performed on a FACS Vantage (BD Biosciences). Analysis of the samples was performed using FlowJo (TreeStar).

Akt kinase assay

Akt in vitro kinase assays were performed using an anti-Akt Ab (Stressgen) or Sepharose-conjugated anti-Akt (N-19; Santa Cruz Biotechnology). Cells were stimulated with plates directly coated with anti-CD3 Abs or coated with streptavidin (Pierce) and then biotinylated anti-CD3 Abs (BD Pharmingen).

Western blotting

Western blots were performed as previously described (16). The following primary Abs were used: rabbit anti-p85α Z-8, rabbit anti-p110α H-201, goat anti-p110α C-17, rabbit anti-p110α N-20 (Santa Cruz Biotechnology), rabbit anti-p85α (Upstate Biotechnology), and mouse anti-c-Myc 9E10.

Competitive adoptive bone marrow transfer

Bone marrow from C57BL/6 Ly5.1 and p110\(_{\text{ABD}}\) mice was depleted of CD3\(^+\) cells by MACS (Miltenyi Biotec), mixed at a 1:1 ratio, and injected into recipient Thy.1.1 mice that had been lethally irradiated with two doses 3–4 h apart. Mice were maintained on oral antibiotics from 3 days before transfer until the time of analysis, at which point animals were euthanized, and the thymi, spleens, and mesenteric lymph nodes were analyzed by flow cytometry.

Results

The p110\(_{\text{ABD}}\) transgene binds to endogenous p85 and induces constitutive PI3K activity in thymocytes

We generated several lines of mice that express the p110\(_{\text{ABD}}\) transgene, consisting of aa 1–108 of bovine class I\(_{\text{A}}\) catalytic subunit p110α. This ABD mediates the association of p110α with p85 adaptor subunits. Transgene expression is driven by the lck proximal promoter, which directs expression to thymocytes (Fig. 1A) (14), and the 9E10 c-Myc epitope was included at the N terminus for Ab recognition.

We could easily communoprecipitate p85α with p110\(_{\text{ABD}}\) via recognition of the Myc tag by the 9E10 Ab (Fig. 1D), but we observed decreased levels of endogenous full-length p110α (Fig. 1C). Because monomeric p110α has been demonstrated to have a shorter half-life than heterodimeric p85/p110 in vitro (17), this suggests that p110\(_{\text{ABD}}\)-mediated sequestration of p85 subunits allows endogenous p110α to exist as an unstable monomer (Fig. 1B). Monomeric p110 subunits have constitutive lipid kinase activity, and indeed, we observed the constitutive activation of Akt, a well-known downstream effector of PI3K, in thymocytes of mice expressing the p110\(_{\text{ABD}}\) transgene (18). As shown in Fig. 1E by an in vitro kinase assay of Akt immunoprecipitates, unstimulated p110\(_{\text{ABD}}\) thymocytes displayed the same maximal level of Akt activity that was induced in nontransgenic thymocytes after 2 min of stimulation with plate-bound anti-CD3 Ab.

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The p110\(_{\text{ABD}}\) mice exhibit increased numbers of mature SP thymocytes

The thymi of p110\(_{\text{ABD}}\) animals had normal cellularity, but significantly increased percentages and total numbers of the more mature thymic subpopulations, e.g., CD3\(^{\text{high}}\)CD4/CD8 SP thymocytes. Fig. 2A shows representative examples of transgenic and NLC thymy from three lines of mice, demonstrating that each of these populations was nearly doubled in p110\(_{\text{ABD}}\) animals, with a slight, concomitant decrease in the DP compartment. The increase in mature SP T cells in the thymus as a result of p110\(_{\text{ABD}}\) expression clearly indicates that the level of PI3K activity can affect T cell development. In contrast, the percentage of DN cells and the percentage of mature thymic subpopulations, e.g., CD3\(^{\text{high}}\)HSAlow SP thymocytes, significantly increased percentages and total numbers of the more mature thymic subpopulations, e.g., CD3\(^{\text{high}}\)HSAlow SP thymocytes.
class I-restricted TCR transgenic mice expressing p110<sub>ABD</sub> (manuscript in preparation).

A variety of mechanisms could account for increased SP populations in the thymus. PI3K activity could improve DP survival, making it easier for the developing cells to express a good TCR and be positively selected; it could improve positively selecting signals, rescuing cells whose TCR react with very low affinity with self-MHC-peptide; it could inhibit negative selection, allowing cells that would normally be deleted to mature; or it could alter emigration of mature thymocytes to the periphery. Although we did not observe any differences in the intracellular location of SP thymocytes or alterations in the expression of CD62L (data not shown), we found that SP thymocytes of p110<sub>ABD</sub> animals exhibited a significantly higher fraction of the most mature SP

FIGURE 1. The expression of p110<sub>ABD</sub> induces constitutive Akt activity and potentiates TCR-induced Ca<sup>2+</sup> flux. A, Diagram of the transgenic construct used to generate p110<sub>ABD</sub> mice. B, Model of p110<sub>ABD</sub> function. In normal cells the adaptor p85 is bound to WT p110 kinase. In thymocytes from p110<sub>ABD</sub> transgenic mice, the ABD from p110 competes with the full-length endogenous p110 for the adaptor p85. Free p110 is catalytically active and unstable. C, Western blot analysis to detect expression levels of WT, full-length p110<sub>α</sub> in thymocytes from NLC and p110<sub>ABD</sub> transgenic mice. D, p85<sub>α</sub> can be coimmunoprecipitated with p110<sub>ABD</sub> via the N-terminal c-Myc tag of the transgenic protein by the 9E10 Ab. Immunoprecipitations were performed with 3 × 10<sup>7</sup> thymocytes/lane. E, In vitro kinase assay of Akt immunoprecipitates from NLC and p110<sub>ABD</sub> thymocytes, unstimulated or stimulated with plate-bound anti-CD3 (5 mg/ml) for 2 or 5 min. F, Thymocytes from NLC and p110<sub>ABD</sub> animals were loaded with Indo-1 and stained with CD4 and CD8, then stimulated with different concentrations of anti-CD3 plus cross-linker (goat anti-hamster; 5.0 μg/ml). The graphs show the mean ratio of Indo-1 fluorescence at 395 and 500 nm for DP thymocytes. Two representative experiments of five are shown. Gray arrow indicates addition of anti-CD3 at different concentrations at 30 s; black arrow indicates addition of 5.0 μg/ml goat anti-hamster at 5 min.
FIGURE 2. Mature, CD3^{high} and HSA^{low} SP thymocyte populations are increased in p110_{ABD} mice. A, Flow cytometric analysis of thymocytes from 8-wk-old mice from three p110_{ABD} transgenic lines: A19516, A19416, and A19415. Thymocytes were stained with CD8-FITC, CD4-PE, and CD3-allophycocyanin. Shown are CD4/CD8 dot plots and a CD3 histogram. The percentage of cells in each region is indicated. The total number of cells per thymus (×10^{6}) is indicated in the upper right corner of the CD4/CD8 dot plot. The gray histogram represents NLC, and the empty black line indicates p110_{ABD}. The percentages of cells in the CD3^{high} gate are represented as control/transgenic. B, The CD4 SP vs CD8 SP ratio of CD3^{high} thymocytes is unaltered by p110_{ABD} expression. Cells were stained, and histograms are represented as described in A. C, p110_{ABD} SP thymocytes are preferentially HSA^{low} compared with NLC. Thymocytes were stained with CD8-FITC, HSA-PE, CD4-CyChrome, and CD3-allophycocyanin.
The expression of p110ABD delays the appearance of peripheral T lymphocytes

In adult p110ABD mice, peripheral T numbers are the same as in NLC animals (data not shown). However, steady state numbers do not rule out changes in the kinetics or rate of development. Thus, we examined the thymic and peripheral phenotypes of neonatal animals. As shown in Fig. 3A, the total thymus size increased at the same rate in both NLC and p110ABD animals. Likewise, the accumulation of TCR\(^{\text{high}}\) SP thymocytes did not occur until after the first month of life. However, αβ T cells appeared in the spleen (Fig. 3B) and mesenteric lymph nodes (data not shown) more slowly in p110ABD animals compared with NLC. The delay in peripheral colonization was observed in both CD4 and CD8 subpopulations of T cells. In contrast, B cell development and colonization were unchanged.

To verify that this difference is also present in adult thymic development rather than being a phenomenon specific to fetal thymopoiesis, we devised a competitive adoptive transfer scheme to compare the emigration kinetics of wild-type (WT) and p110ABD thymocytes. We injected a 1:1 mixture of mature T cell-depleted bone marrow cells from nontransgenic Ly5.1 Thy1.2 C57BL/6 animals and Ly5.2 Thy1.2 p110ABD animals into lethally irradiated Ly5.2 Thy1.1 recipients. At subsequent time points of analysis, the two transferred populations and host cells were discriminated via Ly5.1 and Thy1.1 expression (Fig. 4A, top row). The efficiency of the engraftment of the two donor populations varied from animal to animal, but within each animal we could compare the development of B6- and p110ABD-derived lymphocytes. As shown in Fig. 4A, thymic colonization occurred at the same rate without any reproducible accumulation of p110ABD SP cells compared with wild-type cells. Likewise, B cells efficiently developed from precursors of both genotypes and colonized the spleen (Fig. 4B) and mesenteric lymph nodes (data not shown) at the same rate. In marked contrast, there was a significant delay in the appearance of p110ABD-derived mature αβ T cells in the periphery; WT T cells were readily observable by 3 wk post-transfer, whereas p110ABD T cells did not appear until after 6 wk. Even by 4 mo post-transfer, the ratio of p110ABD T cells to B cells had not reached the same value as in WT-derived cells. This imbalance in the T:B cell ratio was observable even in animals in which the p110ABD cells engrafted better than nontransgenic cells (see 6 wk point in Fig. 4B). The paucity of p110ABD-derived T cells was not due to an impairment in cell survival, because NLC and p110ABD splenic T cells exhibited the same viability in vitro survival assays (data not shown). The p110ABD thymocytes did mature into competent T cells that could exit and survive normally in the periphery; the exit was simply delayed.

The competitive adoptive transfer experiments, in conjunction with the neonatal observations, conclusively demonstrate an important role for PI3K in regulating the emigration of mature thymocytes into the periphery.

Discussion

Class I PI3Ks are involved in both the regulation of mature T and B cell function and the development of T and B cells (7, 25). Studies using PI3K knockout mice have demonstrated that alterations in both the adaptor (p85\(^n\), p55\(^n\), and p50\(^n\)) and catalytic (p110\(^n\)) (11) subunits of PI3K, profoundly inhibit B cell development. However these studies did not reveal any defects in T cell development. PI3K subunit redundancy, suggested by the residual PI3K activity that can be observed in T cells in some of these knockout mice, may account for these results. Alternatively, PI3K activity may not be as important for the development of T cells as it is for B cells.

We have used an alternative approach, transgenic expression of a gain-of-function mutant (p110ABD) in thymocytes, to analyze the role of PI3K at the transition between immature DP and mature SP thymocytes. This system allows for modification of the pathway exclusively in developing thymocytes, which may be important.
FIGURE 4. p110ABD thymocyte egress is delayed relative to that in WT cells in competitive adoptive transfers. A. Thymic development of WT and p110ABD-derived cells is reconstituted by 3 wk in lethally irradiated recipients. A 1:1 mixture of T cell-depleted bone marrow from WT Ly5.1 Thy1.2 and p110ABD Ly5.2 Thy1.2 mice was injected into Ly5.2 Thy1.1 recipient mice. At the indicated time points post-transfer, animals were killed, and the thymocytes were stained with Thy1.1-FITC, Ly5.1-PE, CD4-CyChrome, and CD8-allophycocyanin. Shown are representative animals from groups of two to five recipients. B. Although WT-derived T cells appear in the periphery of recipient mice by 3 wk post-transfer, p110ABD-derived T cells do not exit the thymus until after 6 wk. Even at 4 mo, T cells account for a smaller fraction of p110ABD-derived cells than of nontransgenic-derived cells. Splenocytes were stained with Thy1.1-FITC, Ly5.1-PE, B220-CyChrome, and TCRβ-allophycocyanin.
given that PI3K activity affects the survival and function of APCs (26–28). Furthermore, the activation levels achieved with our construct are comparable to those induced in normal thymocytes by triggering the TCR/CD3 complex. Our results show that thymocyte-specific overexpression of the ABD of p110α induces constitutive PI3K activity, as measured by induction of Ag-independent Akt activity and by potentiation of TCR-induced calcium mobilization. Phenotypically, the thymi of p110ABD transgenic mice show an accumulation of mature CD3highHSAhigh SP thymocytes. Our results demonstrate that this is at least in part due to a significant delay in their emigration to the periphery.

In vitro expression of p110 in the absence of the adaptor p85 or overexpression of p110 in excess of p85 in cell lines results in constitutive p110 kinase activity, although the half-life of p110 is decreased in these circumstances (17). Therefore, it has been proposed that the association of p85 with p110 inhibits its catalytic activity while increasing its half-life (17, 29). We propose that the p110ABD transgene functions as a gain-of-function mutant by sequestering p85, relieving its repression of endogenous full-length p110α. The ability of our p110ABD construct to coinmunoprecipitate p85 and the decreased levels of endogenous p110α (suggesting a shorter protein half-life) indicate that some fraction of endogenous full-length p110α and perhaps other p110 isoforms are present as catalytically active monomers in resting p110ABD thymocytes. The constitutive activation of Akt in resting p110ABD thymocytes agrees with this interpretation. Furthermore, the activation levels achieved with our construct are comparable to those induced in normal thymocytes by triggering the TCR/CD3 complex.

T cells deficient in Tec kinases have alterations in PLCγ activity and calcium mobilization (30). Thus, PI3K may contribute to the modulation of intracellular calcium levels during T cell development by regulating the activation of Tec kinases. This hypothesis is supported by the p110ABD-induced potentiation of calcium flux responses to TCR ligation. Because RIK lacks the PH domains required for membrane localization mediated by PI3K metabolites (31), Itk is likely to mediate Tec family functions in thymocytes downstream of PI3K.

The expression of p110ABD results in an increase in the percentage and total numbers of mature SP T cells in the thymus, clearly indicating that the levels of activity of PI3K can affect T cell development. Intriguingly, unlike others who observed preferential development to the CD4 SP fate in mice with constitutive PI3K activity, we found that both SP populations were increased ~2-fold by p110ABD expression, and the CD4 vs CD8 ratio of CD3high thymocytes was the same as that in NLC mice. The profound delays observed in the colonization of the periphery by p110ABD thymic emigrants clearly indicates a role for PI3K in the control of SP thymocyte maturation and exit. Previous experiments suggested that an activating PI3K transgene (p65PI3K) increased the emigration of CD4, but not CD8, thymocytes to the periphery (13), in direct contradiction of our observations. It is unclear how these results can be reconciled, although the timing and/or intensity of the effect on PI3K activity may account for the differences. Thymocytes have been shown to respond differently to low vs high concentrations of at least one chemokine, CXCL12/stromal cell-derived factor-1 (32), whose receptor (CXCR4) can activate Tec kinases in thymocytes and mature T lymphocytes (33, 34). Perhaps such a concentration-dependent switch also applies to the PI3K activity induced by the emigration signal.

The different stages of thymocyte development are very strictly correlated with specific zones within the thymus. Many groups have attempted to characterize the complex interplay of chemokine and chemokine receptor expression that guide thymocyte movement from zone to zone. These movements are clearly regulated in part by Gα subunits, because PTX expression in the thymus blocks migration across the corticomedullary junction and into the periphery, resulting in the accumulation of functionally mature SP cells in the cortex. However, Ca2+ flux responses to chemokine ligands are unaffected by the expression of PTX, indicating that not all chemotactic signals are PTX sensitive. The potentiated Ca2+ flux responses of p110ABD thymocytes and the defect in periphery colonization suggest that PI3K is one such PTX-resistant chemotactic signal, consistent with its proposed role downstream of the Gβγ heterodimer. Akt is rapidly activated downstream of PI3K after chemokine treatment, followed by a lower level of sustained activation. This activity requires sustained PI3K activity because it is sensitive to wortmannin treatment poststimulation. Furthermore, multiple classes of PI3Ks have been implicated in both Akt and Tec kinase activation downstream of chemokine receptors; the primary peak response is probably mediated by class IA PI3K, whereas the secondary sustained activity is induced via class IB p85/p110 heterodimers.

Despite the clear role of PTX-sensitive (and probably chemokine-mediated) signals in thymic egress, the molecular identities of the receptors involved are not clear. Two candidates have been proposed according to the model of active chemotaxation into the periphery: CCR7 and shingosine-1-phosphate (SIP1). CCR7 expression is up-regulated in postselection thymocytes by combined Ras/ERK and Ca2+ signals; this is accompanied by the acquisition of responsiveness to CCL19 and CCL21 in vitro (35, 36). However CCR7−/− mice do not have a clear thymic exit phenotype (37). Likewise, the spontaneous mutant paucity of lymph node T cells (philopot), which lacks all hemopoietic CCL19 and CCL21 expression, does not display any thymic phenotype that suggests a defect in the release of mature, naïve T cells from the thymus (38–40). More recently, studies of the immune-suppressant drug FTY720 have indicated that sphingoside lipid signaling may play an important role in multiple stages of lymphocyte trafficking (41). In vivo, FTY720 is metabolized into a SIP analog capable of binding the receptors SIP1 (edg1), SIP3 (edg3), and SIP5 (edg5). SIP1 expression is initially induced after positive selection and is progressively up-regulated during SP thymocyte maturation, and a small percentage (2–4%) of late-stage CD62Lhigh SP thymocytes migrate in vitro in response to SIP (42). SIP1 agonist treatment induces the maturation of SP thymocytes to a CD62Lhigh stage, but prevents exit from the thymus (43). In contrast, SIP1−/− SP thymocytes are predominantly CD62Llow and HSAlow, but fail to fully down-regulate CD69 expression, a final maturation step that appears to be required for thymic egress, because these mice exhibit a profound defect in thymocyte emigration (42, 44).

T cell homing to peripheral lymphoid tissues is normal, because SIP1−/− thymocytes transferred i.v. colonize the lymph nodes and Peyer’s patches of WT host animals (42). We have found no evidence for the involvement of either CCR7 or SIP1 in the emigration defect of p110ABD animals; p110ABD thymocytes express both CCR7 and SIP1 normally, and the in vitro chemotactic responses of SP thymocytes to CCL21 and SIP1 are not reproducibly altered (data not shown).

Given the positive role of PI3K activity in chemotaxis in multiple systems, we should expect to observe enhanced chemotactic responses in p110ABD thymocytes. How do we interpret the defect in p110ABD thymocyte egress in light of these results? To make sense of our findings, we must consider work performed in the slime mold Dictyostelium by Meili et al. (45). Abundant evidence indicates that PI3K and Akt are critical regulators of Dictyostelium chemotaxis (reviewed in Ref. 45). One of the most striking features of chemotactic responses is the ability of cells to convert...
shallow extracellular gradients into steep intracellular gradients of signaling molecules via the highly controlled accumulation of PtdIns(3,4,5)P3 at the leading edge of the cell (46). This polarization coordinates the function of PI3K at the leading edge and phosphatase and tensin homologue at the sides and rear (46). Consequently, Akt is rapidly and transiently recruited to the leading edge of the cell (47). In resting cells, an Akt PH domain-GFP fusion protein is evenly distributed throughout the cytoplasm (47). Quickly following stimulation with chemotacticant, Akt returns to the cytoplasm, a phenomenon referred to as adaptation, by which the cell essentially resets. Chemotaxing cells are thus ready to reorient themselves at each chemotaxis step. This dynamic, highly localized accumulation of Akt appears to be required for cells to respond to directional cues. Efficient chemotaxis requires strictly regulated membrane localization of PI3K products and effectors (47). p110αBD expression may impair thymocyte migration by interfering with the proper spatial organization of the cell membrane. By uncoupling endogenous p110α from adapter subunits, p110αBD induces unregulated accumulation of PtdIns(3,4,5)P3 in the cell membrane. This can be observed by the constitutive activation of Akt in p110αBD thymocytes. This could result in uniform membrane distribution of active Akt that, in turn, inhibits the ability of the thymocyte to properly respond to chemotactic signals.

Alterations of the activity of PI3K and its effectors in thymocytes have also been linked to changes in positive selection (13, 48–51). However, in light of our current results, we think that assessment of its possible role in this process will require a more detailed analysis, because in steady state thymus it is difficult to distinguish between increases in the percentages of SP thymocytes due to improved positive selection and those due to delayed exit into the periphery.

In summary, overexpression of the ABD of p110α in thymocytes induces increased PI3K activity, as measured by the constitutive activity of downstream effector Akt and the potentiation of Ca2+ flux responses to TCR ligation. The increased PI3K activity results in the accumulation of mature SP CD3high thymocytes, specifically those in the late HAStim stage. This accumulation is due at least in part to a defect in thymic egress, as shown by the delayed colonization of peripheral tissues by p110αBD T cells in neonatal mice and adoptive transfer recipients. These results demonstrate that PI3K is an important component of the signaling machinery that controls thymic exit, although the upstream factor that regulates this function does not appear to be either of the potential signals identified to date, CCR7 or SIP.

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