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Phosphatidylinositol 3-kinase improves the efficiency of positive selection

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Abstract

We have generated transgenic mice expressing the amino-terminal fragment of the phosphatidylinositol 3-kinase (PI3K) catalytic subunit ($p110_{ABD}$) in thymocytes. Expression of $p110_{ABD}$ results in constitutive activation of PI3K and in significant increases in the numbers of mature, single-positive thymocytes. We previously reported that the increase in mature cells was in part due to a defect in thymic emigration. In this study we identify another component to this phenotype. Expression of $p110_{ABD}$ results in an enhancement of positive selection, without alterations in thymocyte lifespan or negative selection. Since PI3K can affect activation of Btk, which in turn potentiates calcium fluxes, during B cell development, our results suggest that PI3K could play a role in the regulation of Itk kinases in T cells, and that both cell types share a common signaling network to modulate calcium responses downstream of their antigen receptor.

Introduction

During T cell development, the organism generates a T cell population with an extended repertoire of antigen specificities. This repertoire is molded in the thymus through signals derived from the interaction of TCRs on thymocytes with their ligands, MHC molecules with bound peptides. The majority of thymocytes bear TCRs that do not recognize the MHC molecules present in the thymus, and these cells die relatively rapidly (3–4 days). Those cells bearing a TCR able to interact with self-MHC can receive signals that induce either their differentiation into mature T cells (positive selection) or apoptosis (negative selection). Furthermore, those cells that are positively selected develop into two different lineages, CD4 or CD8, depending on the ability of their TCRs to bind MHC class II or I, respectively. Most of what we understand about these processes has been learnt using genetically modified mice, especially mice engineered to express a re-arranged transgenic TCR (Tg TCR). The majority of double-positive (DP) thymocytes in these mice express the same TCR, which can be detected with specific antibodies, and therefore behave as a relatively homogeneous population. Breeding Tg TCR mice with mice with alterations in different signaling molecules has provided most of our current knowledge regarding the role of different signal transduction pathways in the regulation of the different cell fate decisions during T cell development.

One of the pathways important for both positive and negative selection in the thymus is TCR-mediated Ca^{++} fluxes. Ca^{++}-dependent events, such as transcription of the Nur-77 family of transcription factors, appear to be critical for negative selection: expression of a dominant negative Nur77 transgene in Tg TCR^{+} thymocytes inhibits antigen-dependent negative selection (1). However, over-expression in the thymus of low levels of constitutively active calcineurin, a calcium-regulated phosphatase, does not alter negative selection (2), although calcineurin plays an important role in the transcriptional regulation of Nur77 (3, 4). Calcineurin activation instead improves positive selection (2). Therefore, subtle alteration in Ca^{++} responses may have a bigger impact on positive selection.

TCR-induced Ca^{++} influx in thymocytes can be regulated by Tec family kinases (5, 6). These tyrosine kinases contain N-terminal pleckstrin homology (PH) domains that allow

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regulation of the kinase by Class I phosphatidylinositol 3-kinases (PI3Ks). Class IA PI3Ks consist of catalytic subunits that interact with SH2 domain-bearing adaptor subunits, whereas the Class IB PI3K p110γ utilizes a p101 adaptor subunit and is stimulated by G-protein $\beta\gamma$ subunits. While multiple Class IA subunits have been implicated in T lymphocyte signaling, the p85α and p110α subunits are thought to largely mediate PI3K function in T cells.

We have previously described mice expressing the gain-of-function thymocyte-specific transgene $p110_{ABD}$, which induces constitutive Akt activity and potentiates TCR-induced Ca^{+2} influx. We observed an accumulation of mature, $CD3^{hi}$ single-positive (SP) thymocytes in $p110_{ABD}$ mice, and described that this phenotype was in part due to a delay in the exit of mature thymocytes to the periphery (7). Here we show that the increase in mature T cells in the thymus is also partly due to a specific improvement in positive selection. On the other hand, basal DP survival and negative selection are unaffected by $p110_{ABD}$ expression.

Methods

Generation of $p110_{ABD}$ transgenic mice

The $p110_{ABD}$ construct, consisting of amino acids 1 through 109 of p110α, was obtained from Julian Downward (ICRF, London, United Kingdom). The construct was cloned into the *Bam*HI site of the p1017 vector (8), and the *Not*I fragment purified and injected into (C57Bl/6 $\times$ DBA/2J)F1 zygote pronuclei. Founder mice were identified by Southern blotting of genomic DNA probed with a human growth hormone fragment and subsequently backcrossed with C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, USA). Subsequent progenies were genotyped by dot blot using the hGH probe or by PCR with hGH-specific primers. OT-I, HY and AND transgenic animals were all obtained from Jackson Laboratories. Erk^{sem} mice were the kind gift of Stephen M. Hedrick (UCSD).

Flow cytometry

Bcl-2 intracellular staining was performed with the Cytofix/Cytoperm kit (BD PharMingen, cat. no. 2075KK). Briefly, freshly isolated thymocytes (2×10^6) were suspended in 200 μl Cytofix/Cytoperm and incubated on ice for 30 min. After washing with PBS containing 1% FCS and 0.01% Na-Azide, cells were re-suspended in 80 μl Perm/Wash and 20 μl anti-Bcl-2–FITC or isotype-matched control antibody (BD PharMingen, cat. no. 1502KK). Cells were incubated on ice 30 min, then washed twice with Perm/Wash and re-suspended in PBS/FCS/azide for the analysis.

All surface antibodies were obtained from BD PharMingen or eBioscience (San Diego, CA, USA) and were used interchangeably. Analyses were performed using a Beckton-Dickinson FACSCalibur or a Beckton-Dickinson FACScan. Analysis of flow cytometry data was performed using FlowJo (TreeStar Software, Ashland, OR, USA).

In vitro survival assays

In a 96-well plate, 10^6 thymocytes were aliquoted per well. For antigen-independent assays, each well was re-suspended in 100 μl of DMEM complete with 10% FCS and β-mercaptoethanol (ME). Plates were incubated at 37°C in 5% CO_2 for varying times and then analyzed by flow cytometry. Viable DP thymocytes were determined by forward and side scatter. For dexamethasone sensitivity assays, each well was re-suspended in 100 μl DMEM complete/FCS/β-ME containing 0, 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M dexamethasone (Sigma D1756). Plates were incubated for 12 h and then analyzed by flow cytometry. Viable DP thymocytes were determined by forward and side scatter or Annexin V staining. Both methods gave identical results when tested in parallel.

5-Bromo-2-deoxyuridine-labeling experiments

5-Bromo-2-deoxyuridine (BrdU) (Sigma, B5002) was either provided continuously in drinking water at 0.8 mg ml^{-1} until analysis or, for pulse-chase analysis of SP differentiation in AND $H\text{-}2^{b/d}$ animals, was administered in two intraperitoneal injections, each 1 mg in 200 μl PBS, 3-h apart. BrdU staining was performed in duplicate as follows: cells were stained for surface antigens, washed with PBS and re-suspended in 500 μl of 0.15 M NaCl. Ninety-five percent EtOH (1.2 ml) at -20°C was added dropwise, and the cells were incubated for 30 min on ice. Following a wash with PBS, the cells were re-suspended in 1 ml of 1% PFA/0.01% Tween 20 in PBS and incubated overnight at 4°C. The cells were then centrifuged and directly re-suspended in 1 ml of DNase I solution (50 Kunitz units ml^{-1} in 0.15 M NaCl, 4.2 mM $MgCl_2$, 10 μM HCl; Sigma D4527). After incubation for 30 min at 37°C, the samples were washed and stained with FITC anti-BrdU or isotype control antibody (BD PharMingen).

OT-I deletion assays

In vitro deletion assays were performed as described (9). EL4 cells were irradiated with 3000 R and plated at 5×10^4 per well in round bottom 96-well plates. The SIINFEKL peptide was diluted in DMEM and added to varying concentrations ranging from 10^{-7} to 10^{-13} M. Duplicate samples of 10^6 OT-I or OT-I $p110_{ABD}$ thymocytes were added per well to a final volume of 200 μl and incubated for 18 h at 37°C. Samples were then analyzed by flow cytometry as described for survival assays above.

Results

Phenotype of $p110_{ABD}$ transgenic mice

We have described the generation of the $p110_{ABD}$ transgenic mice elsewhere (7). Briefly, the transgene consists of the adaptor-binding domain from the p110α subunit, and its expression is directed to thymocytes by the *lck* proximal promoter. Expression of this transgene competes with wild-type p110 for the adaptors, and results in constitutive activation of p110, as evidenced by constitutive activation of Akt in resting $p110_{ABD}$ thymocytes, and by an improvement in the ability of thymocytes to achieve maximal Ca^{++} influx in response to sub-optimal concentrations of anti-CD3 antibody (7).

$p110_{ABD}$ mice display a distinctive phenotype: the fraction and numbers of mature thymocyte subsets are nearly doubled in transgenic animals compared with wild type. Figure 1 shows

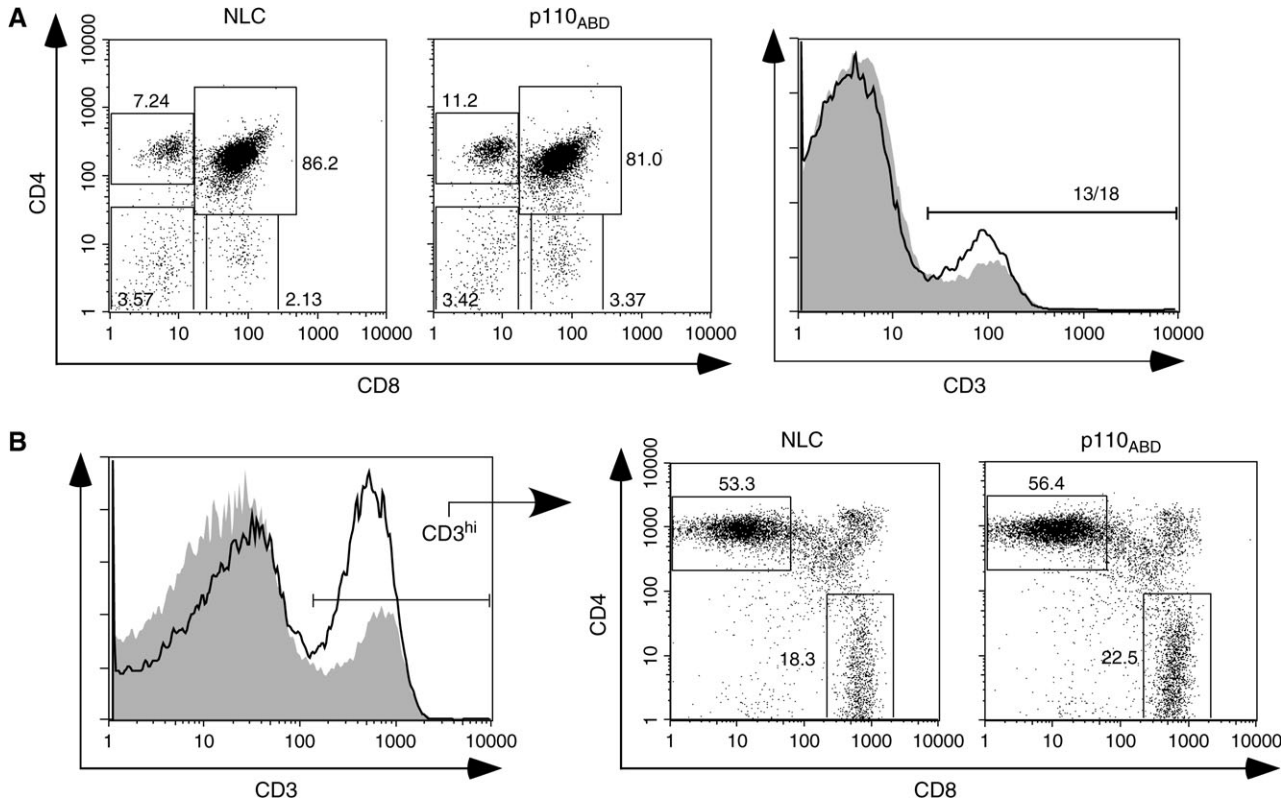

Fig. 1. $p110_{ABD}$ expression increases the percentage of single-positive thymocytes, without altering the CD4/CD8 ratio. (A) Flow cytometric analysis of thymocytes from 8-week old mice from $p110_{ABD}$ transgenic and NLC. Thymocytes were stained with CD8–FITC, CD4–PE and CD3–allophycocyanin. Shown are CD4/CD8 two-color dot plots and a CD3 histogram. The percentage of cells in the different regions is indicated. The percentages of cells in the CD3 high gate are represented as transgenic/control. (B) The $CD3^{hi}$ were gated, and their CD4/CD8 distribution analyzed.

representative examples of transgenic and NLC thymi from one of the four lines of mice generated and demonstrates that both SP compartments as well as the $CD3^{hi}$ fraction are increased by $p110_{ABD}$ expression. All four lines exhibit similar phenotypes, and except where indicated, are used interchangeably throughout this work. We observe a slight, concomitant decrease in the $CD4^{+}CD8^{+}$ DP compartment, but the percentage of $CD4^{-}CD8^{--}$ double-negative (DN) cells is unchanged. When we examined the DN compartment more closely, we could not find any significant changes in the distribution among the DN sub-populations DN1 through DN4 in $p110_{ABD}$ transgenic mice. Also, we did not observe any $p110_{ABD}$-induced change in the numbers of γδTCR+ thymocytes (data not shown). Both αβT cell lineages are similarly affected, as shown by the similar CD4:CD8 ratios observed in the $CD3^{hi}$ compartment of both NLC and $p110_{ABD}$ mice. For the thymi depicted in Fig. 1, these ratios are 2.91 versus 2.51, respectively. As we described previously (7), the SP populations are enriched for cells that are more mature and express low levels of HSA.

$p110_{ABD}$ transgenic animals display a normal peripheral phenotype. NLC and $p110_{ABD}$ mice have similar numbers of peripheral CD4 and CD8 αβT cells and γδT cells in either the spleen or mesenteric lymph nodes. Furthermore, no transgene-induced differences are observed in the periphery of old mice (6 and 9 months), indicating that there is no subtle, accumulative effect. We also examined activation and memory markers on peripheral T cells to see if altered thymocyte development had perturbed peripheral functions. However, we see no changes in the numbers of $CD25^{+}$ $CD69^{+}$-activated T cells or $CD44^{hi}$ $CD62L^{lo}$ memory T cells (data not shown).

We have shown previously that $p110_{ABD}$ expression impairs the exit of mature thymocytes into the periphery. However, effects in the DP compartment could also contribute to the observed phenotype. At the DP stage, PI3K could modulate survival or selection. For example, $p110_{ABD}$ could improve DP survival, making it easier for the developing cells to express a good TCR and be positively selected. Alternatively, it could specifically improve positively selecting signals, rescuing cells whose TCR react with very low affinity with self-MHC+ peptide. On the other hand, PI3K activity could inhibit negative selection, allowing cells that would normally be deleted to mature. All three options are suggested by previous work on PI3K effectors in thymocytes. We therefore examined each process individually to assess the role of PI3K in DP thymocytes.

Possible survival functions of PI3K in DP thymocytes

DP thymocytes have ~3 days in which to receive positive selection signals that induce survival and maturation. Since one of the best-characterized consequences of PI3K-induced

signals in other systems is improved cell survival, we reasoned that it may increase basal DP survival, allowing thymocytes a longer window in which to receive selecting signals rather than directly participating in the signal pathway of positive selection itself.

Because of the loss of Bcl-2 expression, DP thymocytes may die due to the lack of antigenic stimulation (death by neglect). They are also extremely sensitive to physiological stress or corticosteroid exposure. Akt activation can prevent apoptosis in multiple cell types but a similar role for Akt in thymocytes has remained controversial. Whereas both gagPKB and myrPKB expression improved the viability of thymocytes treated with dexamethasone, myrPKB had a negligible effect on survival in the absense of stimulation (10, 11). We thus analyzed death by neglect by culturing thymocytes *in vitro* without antigenic stimulation and assessed cell survival over time. NLC and $p110_{ABD}$ DP thymocytes died at the same rate in these assays (Fig. 2A). We also observed identical apoptotic responses when NLC and $p110_{ABD}$ DP thymocytes were cultured for 6 h with dexamethasone over a range of concentrations (Fig. 2B). Viable DP thymocytes were determined by forward and side scatter or Annexin V staining. Both methods gave identical results when tested in parallel.

Since the survival ability of thymocytes *in vitro* may not be an accurate representation of that *in vivo*, we designed an experiment to examine DP thymocyte lifespan *in vivo*. We monitored the transit of thymocytes through the DP compartment using BrdU. In young animals, the size of the DP compartment remains constant through time; thus, cells will exit the compartment (via death or differentiation) at the same rate as they enter it by β-selection. By supplying animals with BrdU (0.8 mg ml^{-1}) in their drinking water, we label thymocytes *in vivo* during the proliferative burst that occurs at the DN to DP transition. In normal animals, DP thymocytes typically survive for 3 days; therefore, after 1 day of BrdU labeling, one-third of the DP cells should be $BrdU^+$ in wild-type animals. The DP compartments of both $p110_{ABD}$ and NLC mice are completely labeled at similar rates within 3–4 days (Fig. 2C), demonstrating that $p110_{ABD}$ and NLC DP thymocytes have comparable lifespans.

These results were surprising since $p110_{ABD}$ thymocytes have increased basal Akt activity (7), and there is evidence that DP survival can be regulated by Bad and/or $Bcl\text{-}_{XL}$ via Akt. Jones *et al.* (10) observed up-regulation of $Bcl\text{-}_{XL}$ in gagPKB thymocytes that was presumably mediated by reduced association with Bad (10). Furthermore, over-expression of Bad in thymocytes greatly reduces the total number of thymocytes and the percentages of SP thymocytes (12). But in spite of elevated Akt activity, we did not observe changes in Bad phosphorylation at *Ser-136* or $Bcl\text{-}_{XL}$ levels in $p110_{ABD}$ thymocytes. Also, resting $p110_{ABD}$ thymocytes did not exhibit increased nuclear factor-κB (NF-κB) DNA-binding activity compared with NLC, ruling out the up-regulation of survival

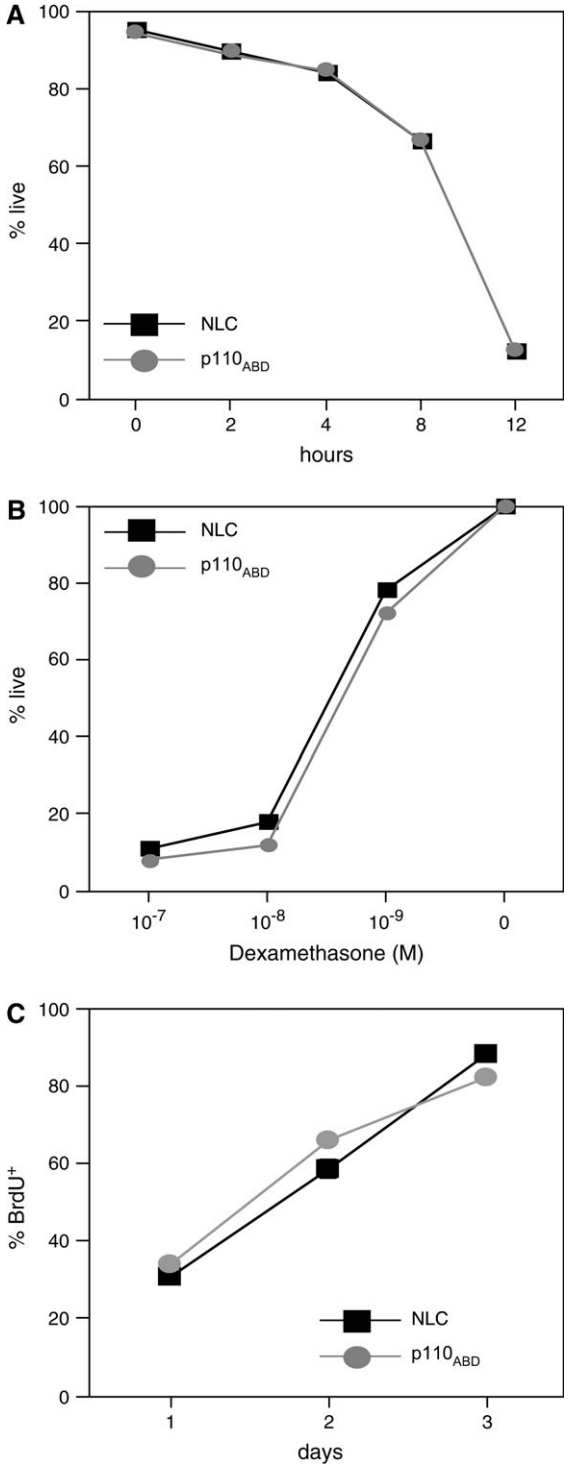

Fig. 2. $p110_{ABD}$ expression does not alter DP survival or lifespan. (A) $p110_{ABD}$ DP thymocytes are as susceptible to 'death by neglect' as NLC. NLC and $p110_{ABD}$ thymocytes were cultured *in vitro* without antigenic stimulation and survival assessed by flow cytometry. (B) NLC and $p110_{ABD}$ SP thymocytes are equally susceptible to steroid-induced apoptosis. Thymocytes were cultured with varying concentrations of dexamethasone for 12 h and then analyzed as in (A). Viable DP thymocytes were determined by forward and side scatter or Annexin V staining. Both methods gave identical results when tested in parallel. (C) NLC and $p110_{ABD}$ DP thymocytes have the same lifespan. Since thymocyte development exists in equilibrium, thymocytes enter the DP compartment at the same rate as which they exit (via death or development). Therefore, when BrdU is supplied in drinking water (at 0.8 mg ml^{-1}), thymocytes integrate BrdU as they become DP during β-selection, and the average lifespan of a DP thymocyte is the time required to completely label the compartment.

genes controlled by this transcription factor (data not shown). These results explain the lack of effect of $p110_{ABD}$ on thymocyte survival, although we do not yet understand the compensatory mechanisms that mediate the regulation of Bad in these cells. However, DP survival is likewise unaffected by the expression of the activating transgene p65PI3K (13), in agreement with our results.

PI3K activity improves thymocyte positive selection in the Tg TCR AND system

To study if the constitutive activation of PI3K effectors could affect positive selection, we crossed the $p110_{ABD}$ transgenic mice with mice bearing the Tg TCR AND. This system has been used in the past to confirm the role of Erk in positive selection (14). The AND TCR recognizes a fragment of pigeon cytochrome C presented by I-E^k molecules. Thymocytes expressing this MHC class II-restricted TCRs are positively selected by I-A^b and I-E^k molecules but not I-A^d (15). Since the $p110_{ABD}$ mice were generated on a C57Bl/6 background, they are H-2^b (I-A^b I-E^o). We bred two lines of $p110_{ABD}$ mice to AND transgenic animals on a H-2^d (I-A^d I-E^d) background to generate progeny with a partially selecting background, H-$2^{b/d}$, in which antigen-presenting cells (APCs) present a low amount of I-A^b that induces sub-optimal positive selection (scored as 25% CD4 SP versus 50% in H-2^b or 10% in H-2^d) (15).

Expression of the $p110_{ABD}$ transgene significantly improves the positive selection of AND-bearing CD4 thymocytes in H-$2^{b/d}$ animals (Fig. 3A). The percentage of CD4 SP thymocytes is increased from an average of 29.2% in AND H-$2^{b/d}$ mice to 41.6% in AND H-$2^{b/d}$ $p110_{ABD}$ animals from the $p110_{ABD}$ 19416 line (n = 21 and n = 24, respectively). Similarly, in animals from line 15669, the CD4 SP fraction is increased from 21.8% (n = 18) to 32.5% (n = 16). As in non-TCR transgenic mice, the HSA^{lo} compartment is especially enriched in animals expressing $p110_{ABD}$. CD4 SP HSA^{lo} thymocytes account for only 6.3% of the thymus in AND H-$2^{b/d}$ mice, but they constitute 18.1% of the thymi of AND H-$2^{b/d}$ $p110_{ABD}$ mice from line 19416. In line 15669-derived mice, the proportions are 7.1% and 17.9%, respectively. The total numbers of thymocytes are decreased in AND H-$2^{b/d}$ $p110_{ABD}$ animals ($19.7 \pm 6.9 \times 10^7$ versus $37.5 \pm 1.5 \times 10^7$), a phenomenon that was also observed previously in AND H-$2^{b/b}$ Erk^{sem} animals (14). Our results suggest that expression of $p110_{ABD}$ dramatically improves AND CD4 SP selection in the H-$2^{b/d}$ background, almost restoring the levels observed in an optimal selecting background, H-$2^{b/b}$.

However, the higher percentages of CD4 SP thymocytes observed in AND H-$2^{b/d}$ animals expressing $p110_{ABD}$ could also be due to the delay in thymic exit we have previously described in these mice (7). To specifically examine the short-term generation of SP thymocytes in the AND H-$2^{b/d}$ background, we performed BrdU pulse-chase experiments. A cohort of proliferating cells was labeled with BrdU by intraperitoneal injection and the thymi were analyzed by flow cytometry 5 days later. The graph in Fig. 3(B) displays the fraction of thymocyte sub-populations that were $BrdU^+$ 5 days after BrdU administration. The majority of proliferating thymocytes are those undergoing β-selection into the DP compartment, as described above. Since the average lifespan of a DP thymocyte is 3 days, most of the $BrdU^+$ cells would have left

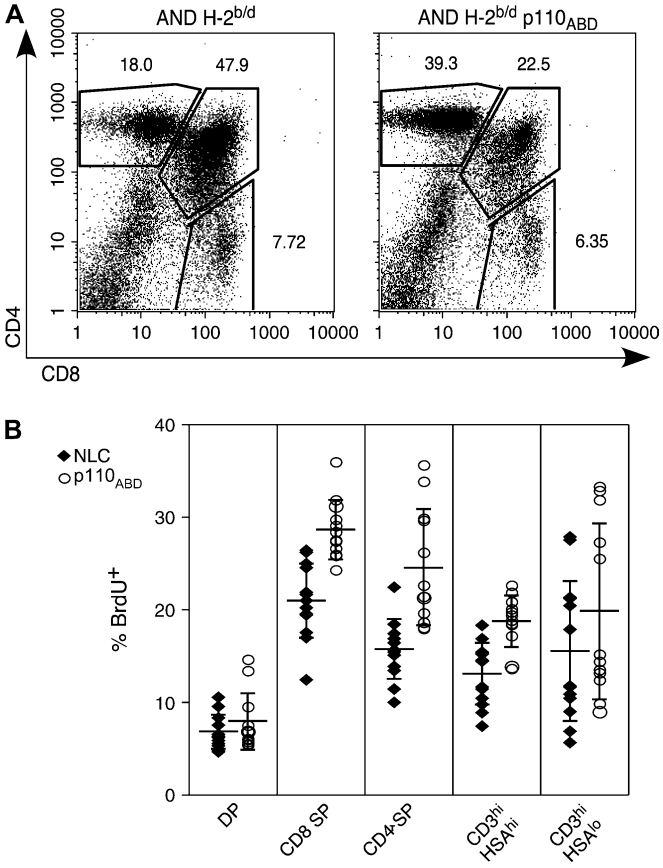

Fig. 3. $p110_{ABD}$ expression improves positive selection of AND Tg TCR thymocytes. (A) The AND Tg TCR is positively selected by I-A molecules but can not direct thymocyte development on I-A: CD4 SP development is sub-optimal in the H-$2^{b/d}$ background. However, expression of $p110_{ABD}$ increased the number of CD4 SP thymocytes in AND mice, indicating improved positive selection. (B) Five days following a pulse labeling of DP thymocytes, more AND H-2 $p110_{ABD}$ mature thymocytes are $BrdU^+$ than AND H-$2^{b/d}$ alone. This reflects more SP and $CD3^{hi}$ thymocytes differentiating from the pool of labeled DP precursors. By contrast, if SP differentiated normally but accumulated in $p110_{ABD}$ animals due to a delayed exit from the thymus, $BrdU^+$ cells would constitute a smaller fraction of SP subsets in AND $p110_{ABD}$ than in AND. Mice were injected twice intra-peritoneally with 1 mg BrdU, 3-h apart. At 5 days, the animals were sacrificed and cells stained for analysis by flow cytometry.

the compartment by 5 days post-label, either by selection or death by neglect. Both AND and AND $p110_{ABD}$ animals have small numbers of $BrdU^+$ DN and DP thymocytes after 5 days. These cells are probably the progeny of early DN subsets that have since differentiated or even newly arrived thymocyte progenitors. However, a greater fraction of CD4 SP thymocytes are $BrdU^+$ in AND $p110_{ABD}$ mice compared with AND littermates. This demonstrates that a greater fraction of the compartment is newly generated each day, indicating improved positive selection. Increased SP proliferation does not account for the higher fraction of $BrdU^+$ cells, as $p110_{ABD}$ expression does not increase SP division (data not shown). $BrdU^+$ cells likewise account for a greater fraction of the $CD3^{hi}$ HSA^{hi} and $CD3^{hi}$ HSA^{lo} populations in $p110_{ABD}$-expressing mice compared with non-transgenic animals after 5 days. BrdU tracking

thus demonstrates that improved positive selection results in greater BrdU labeling of all mature thymocyte subsets.

The effect of p110$_{ABD}$ on positive selection is lineage independent

We observe no skewing of lineage choice in our p110$_{ABD}$ animals. In a non-Tg TCR background, the CD8 SP population is increased the same magnitude as the CD4 SP. As shown in Fig. 1(B), the CD4:CD8 ratio of CD3^{hi} thymocytes in NLC and p110$_{ABD}$ mice was not significantly different: 2.91 versus 2.51, respectively, in the animals shown. We have crossed the p110$_{ABD}$ transgene onto three different Tg TCRs in total, two class I-restricted (HY and OT-I) and one class II-restricted (AND), and we have observed no change in lineage choices (data not shown).

There are significant numbers of CD8 SP thymocytes in all AND H-2$^{b/d}$ mice. Some of these are presumably the 'intermediate' CD8 SP (ISP), immature cells progressing from the DN to DP compartments that up-regulate CD8 prior to CD4. But most are CD3^{hi}, indicating that they are mature cells that have undergone positive selection. Since these animals were Rag$^{+/-}$, these cells may have re-arranged endogenous TCRα chains and been selected by these TCR. Or they may simply be 'mismatched', in that co-receptor expression was not correctly matched to the MHC specificity of the TCR, since AND Rag2$^{-/-}$ precursors can generate TCR^{hi} CD8 SP progeny (16). Interestingly, even though p110$_{ABD}$ expression does not increase the percentage of CD8 SP in AND H-2$^{b/d}$ animals, it does increase the BrdU^{+} fraction of this population in the labeling experiments (Fig. 3A). The increase in BrdU^{+} SP thymocytes was comparable between both lineages. This suggests that p110$_{ABD}$ expression can improve the positive selection of CD8 SP thymocytes even in a system biased to the CD4 fate and argues against a selective role for PI3K in CD4 development only.

Importantly, neither do we observe an accumulation of CD4 SP thymocytes in OT-I p110$_{ABD}$ animals nor in female p110$_{ABD}$ mice expressing the class I-restricted Tg TCR HY. We crossed the OT-I Tg TCR onto two lines of p110$_{ABD}$ mice, 19415 and 15669, and found no significant differences in the CD4:CD8 ratio induced by p110$_{ABD}$ expression. It should also be noted that the populations expressing high levels of the Tg TCR Vα chain, Vα2, exhibit the same CD4:CD8 ratios as for total SP thymocytes. Thus, p110$_{ABD}$ expression does not specifically potentiate CD4 SP development in the class I-restricted Tg TCR systems studied.

The roles of Ras and Erk in p110$_{ABD}$-induced positive selection

The increased positive selection in AND p110$_{ABD}$ animals resembles that observed in AND transgenic animals expressing the Erk2 *sevenmaker* mutant (Erk^{sem}) (14). This mutant is hypersensitive to activation rather than being constitutively active. Intriguingly, Erk was shown to be inhibited in anti-CD3-stimulated T cells treated with wortmannin (17, 18). PI3K-mediated modulation of Erk activation was also suggested by the impaired TCR-induced Erk phosphorylation that is observed in p110δ_{D910A} mutant mice (19). Furthermore, Erk was found to be constitutively phosphorylated in resting PTEN-deficient peripheral T cells (20). We therefore considered the possibility that the p110$_{ABD}$ transgene improves positive selection by enhancing Erk activation.

Resting thymocytes from both NLC and p110$_{ABD}$ mice have the same intracellular levels of phospho-Erk when measured by flow cytometry (Fig. 4A), and both display the same sensitivity to phorbol myristate acetate treatment. We also assessed CD69 expression as a representation of the Erk activity induced by CD3 cross-linking, since Ras activity is sufficient to direct up-regulation of this marker (21). We performed a checkerboard analysis in which thymocytes were stimulated for 3, 9 and 18 h over a range of concentrations. Two analyses of these experiments are shown in Fig. 4(B). There are no detectable p110$_{ABD}$-induced difference in the kinetics of CD69 up-regulation or in the dose-response curve. Thus,

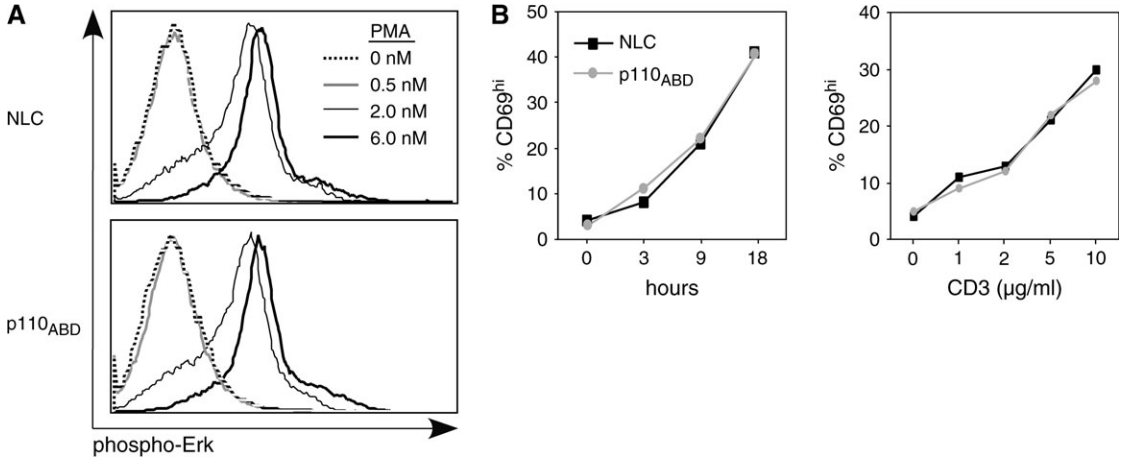

Fig. 4. p110$_{ABD}$ expression does not up-regulate Erk activity. (A) Phorbol myristate acetate (PMA) treatment induces identical Erk phosphorylation in NLC and p110$_{ABD}$ thymoyctes. Freshly isolated thymocytes were stimulated with varying concentrations of PMA for 10 min and analyzed by flow cytometry. (B) CD69 expression serves as a surrogate marker for Erk activity in thymocytes and is up-regulated at the same rate in NLC and p110$_{ABD}$ thymocytes. For the top graph, thymocytes were stimulated with plate-bound anti-CD3 (10 μg ml^{-1}) and CD69 expression assessed by flow cytometry at 3, 9 and 18 h. The bottom graph represents CD69 expression induced by anti-CD3 treatment for 9 h over a range of concentrations.

although $p110_{ABD}$ can potentiate calcium flux responses, and therefore the concomitant DAG production, this does not significantly modify activation of the RasGRP/Ras/Erk pathway in DP thymocytes. We therefore conclude that PI3K can promote positive selection via an Erk-independent pathway.

Negative selection is unaffected in $p110_{ABD}$ animals

Impaired negative selection could also contribute to the observed phenotype of $p110_{ABD}$ mice. We therefore examined the potential effect of the $p110_{ABD}$ transgene in two model systems. For one, we observed deletion of $p110_{ABD}$ thymocytes *in vivo* by viral superantigens (vSAGs). All mouse strains are endogenously infected with a variety of mouse mammary tumor viruses (MMTVs) that express vSAGs ubiquitously. vSAGs constitutively cross-link I-E molecules with specific TCR Vβ segments irrespective of the peptide bound by MHC. This constitutes a strong TCR signal, inducing the deletion of thymocytes bearing such segments (22). C57Bl/6 mice are endogenously infected with MMTV 8 but do not express I-E, and thus retain thymocytes utilizing the Vβ segments recognized by vSAG8, Vβ11 and 12. In addition to MMTV 8, DBA/2J mice carry MMTV 7, and vSAG7 recognizes Vβ6, 7, 8.1 and 9. We crossed the $p110_{ABD}$ mice on the C57BL/6 background ($MMTV8^+$, $I\text{-}E^-$) with DBA/2J mice ($MMTV7^+$, $MMTV8^+$, $I\text{-}E^+$). C57Bl/6 mice have populations of $V\beta6^+$ and $V\beta11^+$ SP thymocytes (6.43 and 4.32% of SP, respectively), but in $I\text{-}E^+$ DBA/2J × C57Bl/6 F1 mice, these populations are deleted by vSAG7 and 8 (Fig. 5A). The $p110_{ABD}$ transgene did not rescue the development of these populations in DBA/2J × C57Bl/6 F1 mice.

SAG-mediated deletion is an extremely stringent form of negative selection, however. If $p110_{ABD}$ exerts a subtle affect upon the signaling thresholds for selection, this effect could be obscured in this system. We therefore utilized an *in vitro* technique that allows dose-response analysis of antigen-mediated deletion of thymocytes expressing the Tg TCR OT-I. The OT-I TCR recognizes an ovalbumin peptide (SIINFEKL) in the context of the MHC class I molecule $H\text{-}2K^b$ (23). The co-culture of OT-I thymocytes with APCs presenting SIINFEKL provides an *in vitro* model for negative selection that is dose dependent (9). OT-I and OT-I $p110_{ABD}$ thymocytes were cultured with APC loaded with different concentrations of SIINFEKL and analyzed after 18 h. DP thymocytes from both OT-I and OT-I $p110_{ABD}$ were efficiently deleted in a dose-dependent fashion, with an LD_{50} of 10^{-10} M peptide. In individual experiments, there appeared to be slight increases in OT-I/$p110_{ABD}$ DP thymocyte survival relative to OT-I thymocytes at high peptide concentrations. However, statistical analyses of the data from multiple deletion assays (n = 14), normalized so that the DP survival rate was expressed as a percentage of the survival in the absence of peptide, showed that the difference was not significant (Fig. 5B). This result confirmed the failure of $p110_{ABD}$ to alter negative selection.

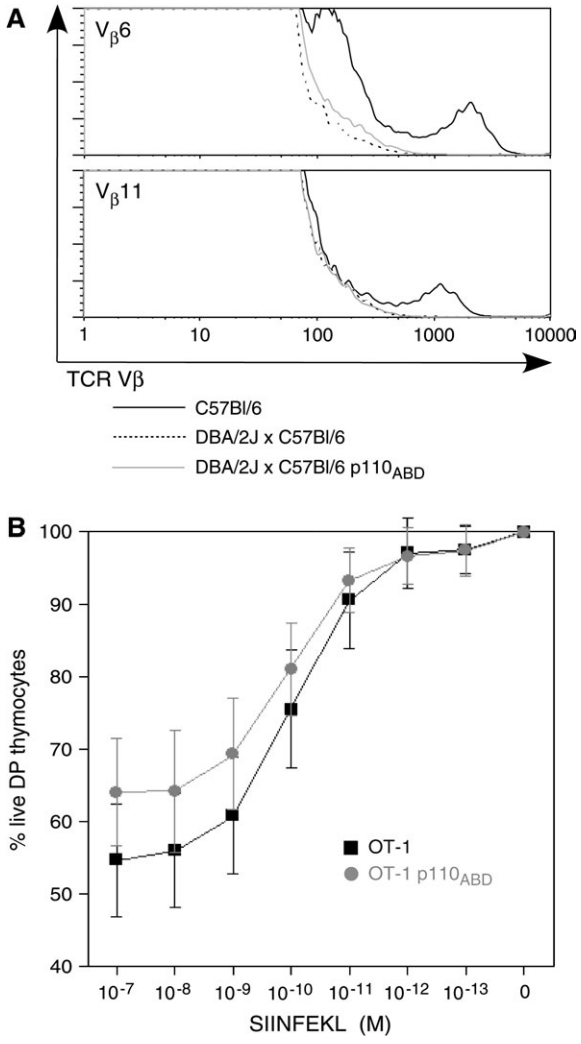

Fig. 5. Negative selection is unaffected by $p110_{ABD}$ expression *in vitro* and *in vivo*. (A) The $p110_{ABD}$ transgene does not protect thymocytes from stringent, SAG-mediated negative selection. $p110_{ABD}$ mice (endogenously infected with MMTV8 but lacking I-E) were crossed with DBA/2J mice ($MMTV7^+$ $I\text{-}E^+$). In the F1 progeny, SAGs 7 and 8 cross-link I-E molecules to TCR $V_\beta6$ and $V_\beta11$, respectively, efficiently inducing deletion of all thymocytes bearing these TCR segments. Expression of $p110_{ABD}$ is not sufficient to rescue the development of these thymocytes in F1 mice. (B) OT-I and OT-I/$p110_{ABD}$ DP thymocytes survive the same in an *in vitro* deletion assay. The $p110_{ABD}$ transgene was crossed onto the OT-I transgenic line expressing a TCR specific for the SIINFEKL peptide, and thymocytes were co-cultured for 18 h with APCs and varying doses of peptide. DP thymocytes will undergo antigen-dependent apoptosis with a frequency related to the concentration of peptide. We find that although OT-I $p110_{ABD}$ DP may survive slightly better than OT-I at high concentrations of peptide, the improvement is not significant when the data from multiple experiments are combined. The graph includes data from eight independent experiments, each performed in duplicate ($n = 14$ for each genotype).

Discussion

Class IA PI3Ks are involved in the regulation of mature T and B cell function (24, 25). Studies using PI3K knockout mice have demonstrated that alterations in both the adaptor (p85α, p55α, p50α) (6, 20) and the catalytic (p110δ) subunits of PI3K (19) 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 observed in T cells in some of these knockout mice, may account for these results.

We have used an alternative approach, transgenic expression of a gain-of-function mutant ($p110_{ABD}$) in thymocytes, to analyze the role of PI3K at the transition between immature DP and mature SP thymocytes. This system allows for the modification of the pathway exclusively in developing thymocytes, which may be important given that PI3K activity affects the survival and function of APCs (26–28). We have previously shown (7) that $p110_{ABD}$ co-immunoprecipitates p85 and decreases the level of endogenous p110α (suggesting a shorter protein half-life). A fraction of the endogenous full-length p110α, and perhaps other p110 isoforms, are present as catalytically active monomers in resting $p110_{ABD}$ thymocytes. The constitutive activation of Akt in resting $p110_{ABD}$ thymocytes agrees with this interpretation. Furthermore, the activation levels achieved with our construct are comparable to those induced in normal thymocytes by triggering of the TCR–CD3 complex.

Expression of $p110_{ABD}$ 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. This phenotype is in part due to alterations in thymic exit in $p110_{ABD}$ mice (7), although other processes may also contribute to it: PI3K 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 or it could inhibit negative selection, allowing cells that would normally be deleted to mature.

An enhancement in survival would be consistent with the role of PI3K in many different systems. The PI3K downstream effector Akt improves survival by phosphorylating the pro-apoptotic Bcl-2 family member Bad and inhibiting its association with Bcl-$_{XL}$, the anti-apoptotic Bcl-2 family member that mediates DP survival (29, 30) It can also increase NF-κB activity (31), another pathway important for survival. Over-expression of Bad in thymocytes greatly reduces the total number of thymocytes and the percentages of SP thymocytes (12), and expression of a constitutively active Akt in thymocytes improves their survival (10). However, in spite of elevated Akt activity, we did not find changes in Bad phosphorylation or Bcl-$_{XL}$ levels in $p110_{ABD}$ thymocytes. Also $p110_{ABD}$ thymocytes did not exhibit increased NF-κB DNA-binding activity compared with NLC. These results explain the lack of effect of $p110_{ABD}$ on thymocyte survival, although we do not yet understand the compensatory mechanisms that mediate the regulation of Bad and NF-κB in these cells. However, DP survival was likewise unaffected by the expression of the activating transgene p65PI3K, in agreement with our results (13).

The increased number of mature T cells in the thymus of $p110_{ABD}$ mice could also be due to improved positive selection. To test this we analyzed positive selection of the AND Tg TCR in a weakly selecting background (H-$2^{b/d}$). This system has been used in the past to confirm the role of MAPK in positive selection (14). Our results show that expression of $p110_{ABD}$ dramatically improved selection in the H-$2^{b/d}$ background to the levels observed in an optimal selecting background, H-$2^{b/b}$. We observed no skewing in CD4/CD8 ratios in our $p110_{ABD}$ animals, by comparison, or appearance of mature CD4 SP bearing class I-restricted TCRs in OT-I/$p110_{ABD}$ animals or in female $p110_{ABD}$ mice expressing the class I-restricted Tg TCR HY (data not shown). This contrasts with recent results that showed appearance of some CD4 SP cells in class I-restricted TCR backgrounds in mice over-expressing p65PI3K (13). Whether this difference is due to the levels of p110 activity or to the use of different class I-restricted TCR strains remains to be determined. By contrast with the positive selection enhancement, we were unable to detect any effects of $p110_{ABD}$ on negative selection, either *in vivo* (using a SAG model or in HY TCR male mice) or using a peptide-induced *in vitro* deletion assay in OT-I mice.

A number of different signaling pathways transduce selecting signals from the TCR to the nucleus and, among them, PI3K activity can regulate activation of the Ras/MAPK pathway and calcium mobilization (24, 25). We directly tested whether expression of $p110_{ABD}$ would affect MAPK activation and calcium mobilization in DP thymocytes. Our results show that $p110_{ABD}$ does not alter the induction of two downstream targets of MAPK, the surface glycoprotein CD69 (21) and the transcription factor Egr (32). Neither their kinetics nor their levels of expression in response to different doses of anti-CD3 antibodies were affected in the presence of $p110_{ABD}$. This suggests that the increased efficiency in positive selection in $p110_{ABD}$ transgenic mice is independent of effects on MAPK. In contrast, expression of $p110_{ABD}$ increased the ability of DP thymocytes to flux Ca^{++} in response to low doses of anti-CD3 (7). The maximal response was not increased, suggesting that the effect of the transgene *in vivo* is more relevant for low affinity TCR–MHC interactions, those that result in positive selection, than for the higher affinity interactions involved in negative selection. Since Ca^{++}-dependent events, such as transcription of the Nur-77 family of transcription factors (1), are critical for negative selection, the lack of effect of $p110_{ABD}$ may be due to a selective role for PI3K in regulating Ca^{++} in response to low intensity stimuli. Although it is possible that the systems we used to determine effects on negative selection are not sensitive enough to detect subtle differences, or that the OT-I *in vitro* deletion cultures do not completely mimic negative selection *in vivo*, over-expression in the thymus of low levels of constitutively active calcineurin, a calcium-regulated phosphatase that regulates expression of Nur77 (3, 4), results in improved positive selection without alterations in negative selection (2). Therefore, subtle alteration in Ca^{++} responses may have a bigger impact on positive selection.

In B cells, PI3K plays a crucial role in the regulation of Btk, a Tec family kinase, by recruiting it to the membrane where it is activated. Activated Btk in turn contributes to PLC-γ activation, leading to increases in intracellular calcium levels (25). In fact, disruptions of the p85α adaptor gene phenocopy the XLA/xid mutants and the $Btk^{-/-}$ mice (6). Thymocytes express high levels of two members of the Tec family of tyrosine kinases (Itk and Rlk) and very low levels of Tec (33). Positive selection is impaired in $Itk^{-/-}$ animals and completely disrupted in $Itk^{-/-}Rlk^{-/-}$ animals (33, 34). Negative selection is apparently normal in Itk null mice but is inhibited in mice deficient for both Itk and Rlk. It remains to be determined whether these effects are due to alterations in calcium responses or to other downstream effectors of Tec kinases (35).

T cells deficient in Tec kinases have alterations in PLC-γ activity and calcium mobilization (36). Thus, during T cell

development, PI3K may contribute to the modulation of intracellular calcium levels by regulating the activation of Tec kinases. Since Rlk lacks the PH domains required for membrane localization mediated by PI3K metabolites (37), Itk is likely to mediate Tec family functions in thymocytes downstream of PI3K. Genetic experiments to test the role of PI3K in regulating Itk are currently being pursued. In agreement with our results presented here, recent studies in the Itk null background observed inhibition of positive selection without alteration of CD4/CD8 lineage commitment (38).

Our results show that during T cell development, PI3K contributes to the regulation of intracellular calcium levels in response to low levels of TCR stimulation and improves the efficiency of positive selection without affecting negative selection or survival. These results, together with experiments in the B cell system where PI3K-mediates activation of Btk which in turn potentiates calcium fluxes, suggest that PI3K could play a role in the regulation of Tec kinases in T cells, and that during development B and T cells share a common signaling network to modulate intracellular calcium levels in response to low intensity signals from their antigen receptor.

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Abbreviations

APC	antigen-presenting cell
BrdU	5-bromo-2-deoxyuridine
DN	double negative
DP	double positive
ME	mercaptoethanol
MMTV	mouse mammary tumor virus
NF-κB	nuclear factor-κB
NIH	National Institutes of Health
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
SP	single positive
Tg TCR	transgenic TCR
vSAG	viral superantigen

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