The Functions of Phosphatidylinositol 3-Kinase in T Lymphocyte Development

Roles in Positive Selection and Thymic Exit

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Susannah Dale Barbee

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Abstract

Phosphatidylinositol 3-kinase (PI3K) is an important regulator of cell survival, proliferation, activation, and migration in multiple organisms and cell types. We have sought to determine how PI3K may regulate T lymphocyte development, a process that entails exquisitely coordinated phases of proliferation, differentiation, and intra-organ movement. We have generated transgenic mice that express a PI3K gain-of-function mutant specifically in thymocytes. The $p110_{ABD}$ transgene constitutes the adaptor binding domain of the PI3K catalytic subunit and this fragment associates with adaptor subunits *in vivo*. $p110_{ABD}$ expression induces constitutive PI3K function, as assessed by the activity of the downstream effector Akt. Furthermore, $p110_{ABD}$-induced PI3K function potentiates Ca^{++} influx induced by sub-optimal crosslinking of the antigen receptor (TCR) on immature thymocytes. Enhancing PI3K activity in developing T cells results in the specific accumulation of late-stage, mature HSA^{lo} $CD3^{hi}$ thymocytes of both lineages. The increased numbers of mature thymocytes can be partly attributed to an improvement in positive selection. This is demonstrated by the ability of $p110_{ABD}$ to promote efficient positive selection of transgenic AND TCR thymocytes in a background that mediates sub-optimal differentiation. The improvement in selection is not biased to the CD4 lineage, since CD4 lineage development is not specifically improved in class I-restricted transgenic TCR animals expressing $p110_{ABD}$. Furthermore, the effect is specific to positive selection since immature thymocyte survival and negative selection are unaffected by $p110_{ABD}$ expression. The increased mature populations are also partly the result of impaired thymocyte emigration. $p110_{ABD}$ T cells colonize the periphery of neonatal animals and irradiated recipients slower than do non-transgenic T cells. The ability of PI3K to regulate positive selection effect is probably due to enhancement of Itk-mediated Ca^{++} influx. By contrast, the role of PI3K in emigration appears to be independent of known chemotactic or adhesive factors and may instead reflect the importance of subcellular organization for chemokine receptor signaling.

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1. Introduction

1.1 T lymphocyte development in the thymus

T lymphocytes are faced with a difficult task. As regulators of adaptive immune responses, they must be able to recognize foreign intrusions of many types. Furthermore, how they recognize foreign challenge will dictate the response mounted by the immune system. T cells survey body tissues via a multimeric receptor complex referred to as the T cell antigen receptor (TCR). The lymphocytes that regulate adaptive responses express a TCR containing α and β chains; another population utilizing γ and δ TCR chains have a different function. αβT cell activation occurs by direct cell-cell contact via the interaction of the TCR with short peptide fragments presented by a MHC heterodimer. T cells can be functionally subdivided into two populations based upon whether they recognize MHC molecules of either class I or II. Class I MHC molecules present peptide fragments derived from intracellular proteins and can thus reveal viral infections, whereas class II molecules instead present peptides derived from extracellular sources such as dying cells or bacteria. TCR are expressed with coreceptor molecules that allow phenotypic discrimination of the two functional populations. The CD8 coreceptor recognizes MHC class I molecules and identify T cells with lytic capabilities. On the other hand, the CD4 coreceptor binds to MHC class II molecules, and CD4 T cells produce growth and inflammatory cytokines when stimulated. These two distinct types of T cells (as well as the γδ T cells) are generated from a common precursor cell in the thymus by a complex and rigorous program of developmental and selective processes.

T lymphocyte development in the thymus has a number of distinct roles. Firstly, T cells must be able to recognize a very broad array of peptide ligands. This diversity of TCR specificity is

accomplished by the rearrangement of small gene fragments to generate both the α and β chains of the TCR. Multiple fragments are available for this process, and the joining mechanism is inherently imprecise, allowing for a nearly infinite number of antigen specificities. However, because of the stochastic nature of this process, TCR capable of interacting with MHC molecules constitute a small fraction of the TCR generated and must be selected from this pool. Secondly, the developing T cells must correctly match coreceptor expression and functional capabilities with the MHC specificity of the rearranged TCR. And third, since each T cell expresses only a single TCR, large numbers of lymphocytes must be generated from few precursors in order to maximize the available repertoire in the periphery. These three goals are accomplished via a highly choreographed developmental process that coordinates rearrangement of the TCR genes, proliferation, and differentiation (reviewed in (1)).

Like all other haematopoietic cells, T lymphocytes derive from multipotent stem cells. Through a mechanism that remains vague, progenitors with T cell potential leave the adult bone marrow and migrate to the thymus via the blood. These initial precursors do not express a TCR or any coreceptors (CD4 or CD8) and are thus referred to as "double negative" (DN: $CD4^-CD8^-$) thymocytes. These DN thymocytes can be further subdivided according to the expression of CD44 and CD25 and to the ability to give rise to multiple lineages of haematopoietic cells. "DN1" cells express CD44 on their surface and subsequently upregulate CD25 expression to become "DN2," which in turn differentiate into $CD25^+44l^{o/-}$ DN3 cells. Whereas earlier DN subsets can develop into NK cells, dendritic cells, or even B cells (DN1 only), DN3 cells can only give rise to T cells. DN3 cells begin to rearrange the β, γ, and δ loci of the TCR, but the mechanism that directs development of the TCRαβ versus TCRγδ lineages remains unclear (2). Successful rearrangement of the TCRβ chain constitutes the formation of a complete gene whose product can be expressed on the surface of the cell. Progression from the DN3 to the DN4

($CD25^{-}44^{-}$) stage accompanies expression of a pre-TCR consisting of the β chain paired with a non-polymorphic pre-TCRα (pTα) chain and the CD3 complex. During the process of "β selection," further TCRβ rearrangement is prevented by allelic exclusion, the thymocytes undergo many rounds of division, and both TCR coreceptors are upregulated.

The resultant $CD4^{+}CD8^{+}$ "double positive" (DP) thymocytes enter a quiescent state and initiate rearrangement of the TCRα locus. Downregulation of Bcl-2 renders the DP thymocytes sensitive to apoptosis, and the failure to successfully rearrange a TCRα chain results in a lack of survival stimuli; these DP cells die by neglect. Alternatively, DP cells that can successfully rearrange a TCRα chain present a complete TCRαβ dimer on the cell surface. The recognition of cognate MHC molecules on antigen presenting cells induces a survival signal referred to as positive selection (3). These DP cells proceed to downregulate a coreceptor to generate either $CD4^{+}$ or $CD8^{+}$ single-positive (SP) thymocytes according to the specificity of TCR for MHC class I or class II molecules (4). Cells that appropriately match coreceptor expression and MHC specificity exit into the periphery.

The primary function of T cell surveillance is the discrimination of "foreign" from "self." A weakness of the manner by which TCR are generated is that potentially auto-reactive T cells can be generated during thymocyte development. Such cells could be activated by healthy tissues and consequently induce autoimmune disease. However, thymocyte development is exquisitely tuned to produce a phenomenon called "central tolerance." The MHC molecules presented to developing T cells within the thymus are loaded with self-peptides; if a DP thymocyte interacts with MHC-peptide with a very high affinity it is instructed to undergo apoptosis in a process referred to as negative selection (5). As a consequence, only DP thymocytes with a TCR that recognizes self-MHC but cannot be fully activated by self-peptide survive and mature, thus

enforcing central tolerance. Few autoreactive T cells do escape central tolerance and mature in the thymus. These cells are subsequently maintained in a quiescent state by peripheral tolerance mechanisms.

Following the molding of the TCR repertoire via positive and negative selection, thymocytes undergo a final maturation process prior to exiting to the periphery. Aside from gross phenotypic alterations, such as the downregulation of CD69 and HSA and the upregulation 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 largely unknown. Mechanisms to explain thymocyte exit have mostly fallen into two central models of active movement: chemorepulsion (or "fugetaxis") from thymic stromal-derived elements or chemoattraction to peripheral signals (6, 7). A third alternative of passive regulation, the loss of responsiveness to thymic-retention signals, has elicited less discussion.

1.2 TCR-coupled signal transduction mechanisms in DP thymocytes

Most stages of thymocyte development are directed by the many signal transduction pathways coupled to the preTCR and TCR complexes. The signaling networks activated are numerous and entail multiple overlapping specificities, functional redundancies, and feedback mechanisms. Figure 1 depicts most of the best-characterized molecules and forward signal pathways involved in TCR signaling in DP thymocytes.

The TCR is always expressed at the cell surface in a complex with multiple CD3 chains due to

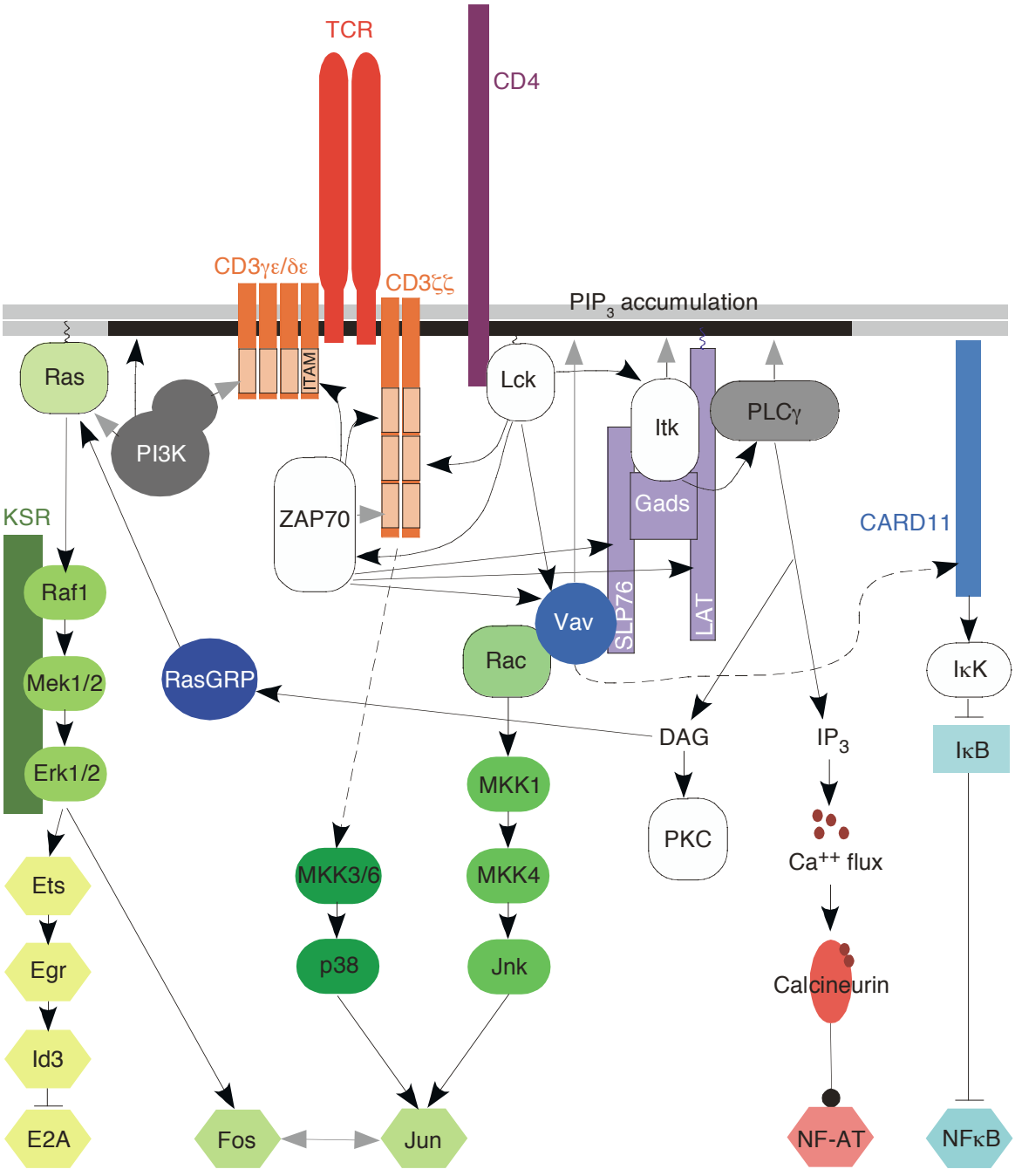

Figure 1. TCR signal transduction in DP thymocytes.
Shown is a simplified diagram of the major signal pathways activated by TCR ligation in thymocytes. Black arrows represent phosphorylation or activation, inverted T's represent inhibition, and lines with back circles indicate activation via dephosphorylation. As yet undetermined pathways are represented with dashed arrows. Grey arrows indicate TCR-induced association.

the interactions of their charged transmembrane domains (8). The TCR/CD3 complex is composed of the TCRαβ heterodimer, CD3γε and CD3δε heterodimers, and a CD3ζ homodimer. During the interaction of the TCR with MHC, CD4 or CD8 coreceptors are recruited to the TCR/CD3 complex. The Src family kinase Lck is associated with the intracellular domain of both coreceptors, but with greater affinity for CD4 (9). Once in the vicinity of the TCR complex, Lck phosphorylates the CD3 chain ITAMs (immunoreceptor tyrosine-based activation motifs). This allows the recruitment and activation of Zap70, which completes phosphorylation of the CD3 ITAMs (10).

Heavily-phosphorylated ITAM domains recruit large "signalsome" complexes via multiple adaptor protein components. One of the most important of the multi-protein complexes recruited by TCR signals is that associated with the membrane-tethered adapter LAT, including the adaptors SLP76 and Gads, Tec family tyrosine kinases (Rlk or Itk), PLCγ1 and Vav (11). ITAM phosphorylation also induces Phosphatidylinositol 3-kinase recruitment to the TCR and subsequent activation (PI3K signals are discussed in greater detail below). PI3K induces local accumulation of PtdIns(3,4,5)P_3 (Phosphatidylinositol(3,4,5)P_3), recruiting pleckstrin homology (PH) domain-containing proteins such as Akt, Tec family members, and PLCγ1. The association of Tec kinases and PLCγ1 with PI3K products may contribute to the colocalization of the LAT-associated signalsome with the TCR/CD3 complex (12). At the TCR, Lck phosphorylates and activates many components of the signalsome, including Tec kinases (13).

Tec kinases can activate PLCγ1, thereby inducing the generation of IP_3 and DAG products which in turn regulate Ca^{++} flux responses and PKC, respectively (14-16). Calcineurin is a calcium-dependent phosphatase that binds Ca^{++} to become activated. Calcineurin subsequently dephosphorylates NF-AT, allowing nuclear translocation and transcriptional activation (17).

TCR-mediated activation of transcription also includes upregulation of NF-κB. NF-κB is normally sequestered in the cytoplasmic by the associated IκB. IKK phosphorylates IκB, thereby targeting it for ubiquitylation and degradation by the proteosome. The transcription factor NF-κB is therefore freed to translocate to the nucleus and activate transcription. Yet another component of the LAT signalsome, Vav, is required for IKK activation in thymocytes and T cells, likely via the membrane-associated protein Card11 (18).

One of the hallmark responses to TCR triggering is the activation of Ras, which can be mediated by the LAT-associated signalsome via two alternative pathways. In DN thymocytes, LAT can recruit the RasGEF Sos via the adaptor protein Grb2 (19). But Ras activation in DP thymocytes appears to depend instead upon the DAG- and PKC-dependent RasGRP, and Ras is therefore induced by LAT-associated PLCγ1 activity (20, 21). Ras activation in turn activates the Raf/Mek/Erk cascade, positively regulating Ets transcription factors and negatively regulating E2A (22). Another MAPK cascade, the MKK1/MKK4/Jnk pathway, is activated by TCR triggering of DP thymocytes. Jnk function is regulated by Vav via the Rac-induced activation of MKK1 (23, 24). The p38 MAPK pathway appears to be an important TCR-induced pathway as well, but the mechanism by which they are coupled remains unknown. Activation of the various MAPKs results in Fos/Jun dimerization to generate the transcriptional activator AP-1. Ultimately, the coordinate activation of these many pathways is integrated into the activation of distinct genetic programs by groups of transcription factors including Ets factors, AP-1, NF-AT, and NF-κB.

1.3 The molecular basis of thymocyte selection

For the last 20 years, one of the central questions of thymocyte development has been about the distinction between positive and negative selection. How do DP thymocytes "know" that they express a potentially autoreactive TCR and should therefore execute an apoptotic program? This question is further complicated by the nature of the signals that mediate selection: both negative and positive selection are mediated by self-derived peptides. Early experimental evidence indicated that thymocyte selection is best described by a qualitative model in which the activation of unique signal transduction pathways directs cell fate, as opposed to a quantitative model which proposes that identical signal transduction pathways downstream of the TCR are activated during both positive and negative selection but to different degrees (5, 25). The picture that has emerged more recently has integrated both of these models and suggests that signal intensity thresholds direct activation of qualitatively different signals (26).

In vitro studies demonstrate that the fate of a DP thymocyte maturing into either the $CD8^+$/MHC class I- or the $CD4^+$/MHC class II-restricted lineage is directed by the nature of the peptide presented: antagonist peptides and even some weak agonists promote maturation whereas strong agonists induce negative selection (27-30). Strong agonists typically bind to the TCR with a K_d of 1-90 μM and a half-life of approximately 10 seconds (31). Minor mutations that convert these peptides into partial agonists correlate with decreased half-lives of the MHC-peptide/TCR complex and loss of antigen-mediated deletion *in vitro* (31, 32). But how do the quantitative differences in TCR interactions translate into different developmental outcomes? Two models have been proposed to answer this question, the threshold and the duration models (5). Simply put, the threshold model posits that weak interactions activate only a subset of the signals induced by high-affinity interactions. According to this scheme, signals required for positive selection

have a lower activation threshold than the pathways that mediate negative selection. For each TCR/ligand interaction, signal transduction pathways that are slowly recruited to the TCR complex may be activated only by the long-lived interactions with agonists and instruct the thymocyte to undergo apoptosis. On the other hand, positive selection has been shown to require sustained interactions with stromal cells, suggesting that an ongoing TCR triggering mediates positive selection. (33, 34). These findings have been incorporated into a duration model, in which high affinity interactions mediate a strong but transient signal. Low affinity interactions instead trigger weak but sustained signals that mediate positive selection. To date a large number of the signals downstream of the TCR have been implicated in positive versus negative selection discrimination (summarized in Table 1). How these models can be applied will be discussed below in the context of each effector that supports either.

The generation of a number of mouse lines expressing transgenic (Tg) TCR has greatly assisted the studies of positive and negative selection. The original studies on agonist and antagonist peptides mediating different selection outcomes were performed in the context of Tg TCR. Also, MHC class I- and class II-restricted Tg TCR that induce different selection outcomes based upon H-2 haplotype and gender have proven especially useful for separating selective processes *in vivo*. Three such Tg TCR that will be discussed further below are the class I-restricted HY and OT-1 TCR and the class II-restricted AND TCR. The antigenic peptide recognized by the HY receptor is encoded on the Y chromosome: CD8 SP thymocytes are positively selected in female animals but negatively selected in males (35). The OT-1 TCR recognizes a peptide derived from chicken ovalbumin, SIINFEKL. A number of altered peptide ligand agonists and antagonists have been characterized for this Tg TCR (29). The AND receptor was cloned from T cells specific for a fragment from pigeon cytochrome C and reacts with varying affinity with class II molecules of different haplotypes. The development of AND Tg TCR^+ CD4 SP is positively

selected by I-A^b and I-E^k molecules but negatively selected by I-A^s (36).

Genetic studies in mice have demonstrated the importance of immediate, TCR-proximal signaling events for DP differentiation. The CD3δ chain appears to be a critical TCR/CD3 component for initiation of positive selection signals, as mice lacking this chain exhibit decreased SP development (37). The role of CD3δ appears to be a structural one, however, since a tailless CD3δ transgene is capable of rescuing the knockout phenotype (37). Furthermore, a similar phenotype is observed in animals expressing TCRα chains with a mutation in the domain that mediates association with CD3δ (CPM) (38). Intriguingly, no negative selection defects have been reported in any of these animals. Most of the TCR-proximal adaptor molecules and tyrosine kinases appear to be equally required for positive and negative selection, however. Both selective processes are completely blocked in animals deficient for Zap70 and in $Lck^{-/-}$ animals expressing a Lck transgene that cannot associate with CD4 or CD8 (4, 77). Lck at least appears to play a dose-related role for both positive and negative selection, since catalytically inactive (dLGKR) and active (dLGF) transgenes partially inhibit and improve positive selection, respectively (22, 39, 40). These results have been interpreted as support for the threshold model of positive versus negative selection. Unfortunately, thymocytes lacking the dominant adaptors at the DP stage, LAT and SLP76, cannot develop beyond the β-selection checkpoint (41, 42). But groups have begun to identify critical domains in these proteins by knock-in or transgenic expression of various point and deletion mutants in the deficient backgrounds. Thymocytes are capable of sub-optimal positive selection when the Gads-interacting domain of SLP76 is deleted, but there is a more stringent requirement for N-terminal tyrosine phosphorylation sites that mediate association with Vav and Itk (42). On the other hand, positive and negative selection of Tg TCR^+ thymocytes are both inhibited in mice deficient for Gads, which is typically associated with both LAT and SLP76 in DP cells (43). Thus TCR-proximal adaptors appear to be required for coupling the

TCR to both positive and negative selection effector pathways.

Numerous studies demonstrate that the Ras/Erk cascade is absolutely required for positive selection. Early work utilizing transgenic mice expressing dominant negative forms of Ras or Mek1 under control of the *lck* proximal promoter indicated that this signal cascade is essential for positive selection but dispensable for negative selection (44-46). Similarly, expression of a dominant-negative form of Raf inhibits positive selection of female HY thymocytes whereas the constitutively active, oncogenic form, vRaf, improves the positive selection of HY^{+} thymocytes in male animals (47). SP thymocytes of both CD4 and CD8 lineages are decreased by roughly 50% in Erk-$1^{-/-}$ mice, as well as $CD3^{hi}$ thymocytes and $CD69^{+}$ cells, despite normal thymus size (48). On the other hand, positive selection is improved in mice expressing the hypermorph Erk^{sem} (49). Scaffolding proteins are also important in the regulation of positive selection by the Erk pathway: retroviral-mediated over-expression of Ksr (for Kinase Suppressor of Ras) inhibits positive selection in reaggregate fetal thymic organ culture (FTOC) experiments (50). We currently theorize that Erk mediates positive selection through the Egr/Id3/E2A transcription factor network. This is suggested by observation of improved positive selection when wild-type Egr1 is overexpressed in thymocytes (51). Furthermore, SP development is inhibited in $Egr1^{-/-}$ and $Id3^{-/-}$ animals and in mice overexpressing the Egr inhibitor Nab2 (52, 53, E. Tse and JAI, unpublished results). Id proteins are repressor partners for E2A proteins, and indeed $E47^{-/-}$ mice exhibit improved positive selection of both class I- and class-II-restricted Tg TCR thymocytes (54). Notably, the effect is dosage-dependent, as the improvement was less pronounced in $E47^{+/-}$ animals, a result that can be interpreted as evidence for quantitative models of TCR signaling. The selection-mediating genes upregulated by Erk pathway activation remain to be identified.

One of the most striking features of the genetic studies of Ras/Erk in thymocytes is the absolute

restriction of effects to positive selection. Negative selection is completely unperturbed in all *in vivo* systems examined. A role for this pathway in negative selection is only suggested by studies using pharmacological inhibitors, such as Mek-specific inhibitors PD98059 and UO126. These studies do replicate the positive selection phenotypes of the genetically-modified systems: both PD98059 and UO126 inhibit CD4 lineage maturation and final TCR upregulation to TCR^{hi} in both lineages (55). On the other hand, some groups report inhibitor-mediated negative selection effects (56, 57) whereas others do not (58). It is possible that effects on negative selection are due to nonspecific inhibition of other molecules. Although resolution of this issue in the near future seems unlikely, popular opinion holds that Ras signals are not involved in negative selection. The Ras pathway is considered to be a qualitatively distinguished signal. The positive selection-specific functions of Erk have been offered as evidence for the duration model of selection in conjunction with the observations of TCRα-CPM mutant thymocytes. Werlen *et al*. observe that Erk is activated only transiently by high-affinity ligands, whereas low affinity ligands induce a sustained Erk activity (38). Furthermore, the sustained activity is abrogated in TCRα-CPM cells, but transient Erk activation in response to high-affinity signals is normal.

Most studies indicate that the Raf/Mek/Erk cascade is not the only Ras-induced pathway required for positive selection. Thymocyte development in dnRas transgenic mice cannot be rescued by the expression of activated Mek or Erk^{sem} transgenes (22, JAI unpublished results, see Fig 11C). The missing effector(s) remain to be identified. Thus far most of the other Ras-related signaling molecules that have been identified are instead situated upstream of Ras. For example, RasGRP is suspected to be the dominant Ras activator in DP thymocytes because the phenotype of $RasGRP^{-/-}$ animals duplicates that of dnRas mice (59, 60).

The p38 and Jnk MAPK cascades are also activated downstream of the TCR and positively

regulate the c-Fos and c-Jun components of AP-1. In contrast to Erk, these MAPKs may be important in positive and/or negative selection, but experimental data has proven frustratingly contradictory. Thymocyte development is grossly normal in mice deficient for either of the p38-specific MAPKKs, MKK3 or MKK6 (61, 62). But apoptosis measurements (caspase activity and DNA fragmentation) are decreased in the $MKK6^{-/-}$ cells considered to be undergoing negative selection, $TCR\alpha\beta^{int}$ $CD69^{lo}$, in the absence of MKK6 (61). Treatment with the p38-specific inhibitor SB203580 enhances SP generation in both CD4 and CD8 lineages (58), but it is unclear whether this is due to enhanced positive selection or impaired negative selection. Reducing p38 activity by expressing dominant-negative MKK6 and MKK3 transgenes can improve the positive selection of both AND and HY Tg TCR thymocytes, but negative selection was not examined with these Tg TCR (63). On the other hand, SB203580 rescues DP thymocytes deleted in FTOC by anti-CD3 treatment and in negatively-selecting HY Tg TCR male mice (58). Consistent with a role in negative selection, over-expression of MKK6 significantly reduces the number of DP thymocytes in FTOC and almost blocks mature SP development (58)

Jnk has also been implicated in negative selection although, unlike p38, it is not constitutively active in DN and DP thymocytes. Jnk is activated by the MAPKKs MKK4 and MKK7, perhaps synergistically (64). Transgenic mice expressing a dominant-negative MKK4 under the control of the *lck* proximal promoter have normal Fas-mediated deletion of DP thymocytes despite a lack of Jnk activity (65). However, Jnk activation by PMA and Ionomycin remains unaltered in dMKK4 thymocytes, suggesting that MKK4 is dispensable for induction of Jnk by the TCR (65). Fas-mediated deletion of $MKK7^{-/-}$ thymocytes is also normal, although the activity of Jnk was not assessed in the thymocytes of these mice (64). Expression of a dominant-negative form of Jnk (dnJnk1) provides partial protection from antigen-specific deletion of DP thymocytes in Tg TCR mice but does not influence positive selection (66). $Jnk1^{-/-}$ T_H cells and splenocytes

hyperproliferate in response to TCR ligation, but no effects have been characterized in thymocytes (67).

The Jnk pathway may be activated in thymocytes by the Rho family member Rac, which is activated by the GTP exchange factor Vav. Despite having very few thymocytes, $Vav^{-/-}$ thymii do contain small populations of SP thymocytes and generate mature, peripheral T cells (68, 69). However crosses to class I- or class II-restricted Tg TCR mice reveal a severe inhibition of positive selection (69, 70). $Vav^{-/-}$ thymocytes are also more sensitive to SAG-mediated negative selection (68) and to peptide-induced apoptosis *in vitro* (70). Intriguingly, expression of a constitutively active Rac transgene in thymocytes converts positive selection to negative selection: L61Rac1 prevents the development of CD8 SP in female HY animals (71). This selection conversion requires TCR/ligand interaction, however, as class I-restricted Tg TCR thymocytes are positively selected in L61Rac1 $\beta_2m^{-/-}$ mice (71).

Ca^{++}-dependent events, such as transcription of the Nur77 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 (72). However over-expression in the thymus of low levels of constitutively active calcineurin, a calcium-regulated phosphatase, does not alter negative selection (73), although calcineurin plays an important role in the transcriptional regulation of Nur77 (74, 75). Calcineurin activation instead improves positive selection (73). Therefore, subtle alteration in Ca^{++} responses may have a bigger impact on positive selection.

Genetic modification of lipid signaling molecules and effectors has also revealed a role for PtdIns signals in the selection process, which will be discussed below.

Table 1. Proteins implicated in positive and/or negative selection

Protein	Genetic modification	Positive selection	Negative Selection	References
TCR				
CD3δ	CD3δ$^{-/-}$	inhibited	n.e.	(37)
	CD3δ$^{-/-}$ + tailless CD3δ Tg	normal	n.e.	(37)
	TCRα-CPM mutant (cannot bind CD3δ)	inhibited	normal	(38)
CD3γ	CD3γΔITAM	inhibited	normal	(40)
Adaptor Molecules				
Cbl	Cbl-/-	improved		(76)
Gads	Gads$^{-/-}$	inhibited	inhibited	(43)
Slp76	Slp76$^{-/-}$ + Slp76Δ224-244 Tg (cannot bind Gads)	inhibited	n.e.	(42)
	Slp76$^{-/-}$ + Slp76 Y3F Tg (mutation of N-terminal binding sites)	inhibited	n.e.	(42)
Tyrosine Kinases				
Zap70	Zap70$^{-/-}$	blocked	blocked	(77)
	Zap70$^{-/-}$ + inactive ZAP-70 Tg	inhibited/blocked	inhibited	(78)
Lck	dLGKR (inactive) Tg	inhibited	inhibited	(22, 39)
	dLGF (active) Tg	improved	improved	(22, 40)
	Lck$^{-/-}$ + LGCA Tg (cannot bind CD4/8)	blocked	blocked	(4)
Ras and MAPKs				
RasGRP	RasGRP$^{-/-}$	inhibited/blocked	normal	(59, 60)
Grb2	Grb2$^{-/+}$	normal	inhibited	(79)
Ras	dnRas Tg	inhibited	normal	(45, 46)
Raf	dnRaf Tg	inhibited	??	(47)
	vRaf (active) Tg	improved	??	(47)
Mek	dMek Tg	inhibited	normal	(44, 45)
	dnRas + dMek Tg	blocked	normal	(45)
	dnRas + actMek Tg	inhibited	n.e.	(22)
	dMek + actMek Tg	partially restored	n.e.	(22)
Erk	Erk1$^{-/-}$	inhibited	n.e.	(48)
	Erk2^{sem} (hypersensitive) Tg	improved	normal	(49)
	dnRas + Erk2^{sem} Tg	inhibited	n.e.	JAI, unpublished results;see Fig.11
Ksr	Ksr retroviral over-expression	inhibited	n.e.	(50)
MKK6	MKK6$^{-/-}$	normal	inhibited?	(61)
	dnMKK6 + dnMKK3 Tg	inhibited	n.e.	(63)
	MKK6 retroviral over-expression	n.e.	improved	(58)
Jnk	dnJnk1	normal	inhibited	(66)

(continued)

n.e.= not examined

Table 1. Proteins implicated in positive and/or negative selection (cont.)

Protein	Genetic modification	Positive selection	Negative Selection	References
Rho family-related				
Vav	$Vav^{-/-}$	inhibited/blocked	improved	(68-70)
Rac	L61Rac-1 (active) Tg	converted to negative	n.e.	(71)
Ca^{++}-related				
Calcineurin	Calcineurin P2bΔ (active)Tg	improved	normal	(73)
CaMKIV/Gr	$CaMKIV/Gr^{-/-}$	inhibited	normal	(80)
Nur77	dnNur77 Tg	n.e.	inhibited	(72)
Lipid Signaling				
PI3K	$p65^{PI3K}$ (active) Tg	CD4 only improved	n.e.	(81)
	$p110\gamma^{-/-}$	CD4 only inhibited	n.e.	(81)
PTEN	$PTEN^{flox/-}$	inhibited	inhibited	(82)
SHP-1	me^{v} ($SHP\text{-}1^{-/-}$)	improved	improved	(83, 84)
	dnSHP-1 Tg	improved	improved	(83)
Tecs	$Itk^{-/-}$	inhibited	varies	(85-87)
	$Itk^{-/-}$ $Rlk^{-/-}$	converted to negative	inhibited	(86)
	Rlk Tg over-expression	converted to negative	n.e.	(16)
	$Itk^{-/-}$ + Rlk Tg	partially rescued	n.e.	(16)
Transcription Factors				
Egr	Egr1 Tg over-expression	improved	n.e.	(51)
	$Egr1^{-/-}$	inhibited	normal	(52)
	Nab2 Tg over-expression (Egr inhibitor)	inhibited	n.e.	E. Tse and JAI, unpublished results
E2A	$E47^{-/-}$	improved	normal	(54)
Id3	$Id3^{-/-}$	inhibited	inhibited	(53)
NF-κB	super-inhibitory IκBα Tg	inhibited	normal	(88)

n.e.= not examined

1.4 Chemokines and thymocyte trafficking

The sequential stages of thymocyte development are highly coupled to the intrathymic environment. The thymus itself is grossly organized into a cortex and a medulla, with subcortical zones along the periphery of the organ. Each differentiation step is the result of an interaction between the T cell precursor and a specialized stromal cell. For example, positive selection is initially induced by the interactions of DP thymocytes with epithelial cells in the cortex, whereas medullar dendritic and $CD30L^{+}$ epithelial cells mediate efficient negative selection (89). As a result, thymocytes are almost always moving in the thymus, constantly surveying the stromal cell types in their vicinity. Recently a great deal of work has shown that this process is mediated by multiple small, soluble molecules called chemokines and their cognate G protein-coupled receptors (GPCR).

Chemokine receptors are seven transmembrane, serpentine receptors coupled to heterotrimeric G proteins. Receptor activation results in the exchange of GTP for GDP by the G_{α} subunit and the subsequent dissociation into G_{α} and $G_{\beta\gamma}$ subunits, which likely mediate separable functions. Multiple signal transduction pathways are activated downstream of these receptors in T cells, including (but not limited to) JAK-STAT, focal adhesion kinase (FAK), MAPK, PI3K, and Ca^{++} influx (reviewed in (90)). The physiological consequence of chemokine binding is the polarization of the cell and accumulation of chemokine receptors at the leading edge, followed by oriented cell movement (91). Accordingly, much research has focused on the mechanism by which these receptors direct cytoskeletal remodeling. CXCL12 treatment of Jurkat T cells induces the phosphorylation of several proteins associated with the cytoskeleton, FAK, Cas (Crk-associated substrate), WASP, and Nck (92). In fact, most of what is known about chemokine receptors in T cells has been derived from the study of CXCL12 (SDF-1) and its receptor CXCR4

(best known as the coreceptor for HIV infection (93)). In the MOLT4 transformed T cell line, CXCL12 treatment induces the rapid phosphorylation of CXCR4 by the Janus kinases JAK2 and JAK3 (94). JAKs appear to be important for CXCR4 signaling since the JAK-specific inhibitor AG490 blocks cell movement and Ca^{++} flux responses, but these effects may be mediated by an alternative pathway since CXCL12 induces minimal nuclear translocation of STATs (94). JAKs may instead be important for cytoskeletal remodeling, as AG490 treatment of a haematopoietic progenitor cell line blocked CXCL12-induced FAK phosphorylation (95). However, the most prominent consequence of chemokine stimulation by far is the activation of PI3K, which will be discussed in further detail below.

Distinct stromal cells types in the thymus express various chemokines whereas chemokine receptor expression by thymocytes is developmentally regulated (Fig. 2) (96). DN pro-T cells enter the thymus via venules in the cortico-medullary junction (CMJ) and rapidly migrate across the cortex and to subcapsular zone. The earliest thymic immigrants express CXCR4, the receptor for CXCL12 (97-99). The CXCL12/CXCR4 signal appears to assist in the specific recruitment of T precursors into the thymus, since deletion of CXCR4 induces a complete block at the DN stage (99). CXCR4-deficient thymocytes can enter the thymus, but remain at the CMJ, and $CXCL12^{-/-}$ thymocytes likewise accumulate at the DN stage (99, 100). Intriguingly, CXCL12 expression is restricted to the cortex, and CXCR4 expression is downregulated on DP thymocytes undergoing positive selection (97, 99, 100). By contrast, DP thymocytes upregulate CCR9 expression, and its ligand, CCL25 (TECK) is expressed throughout the thymus (97). CCR9 appears to be completely dispensable for thymocyte development, however, since T cells are produced normally in $CCR9^{-/-}$ mice (101).

Positive selection induces the upregulation of both CCR4 and CCR7: CCR7 expression requires

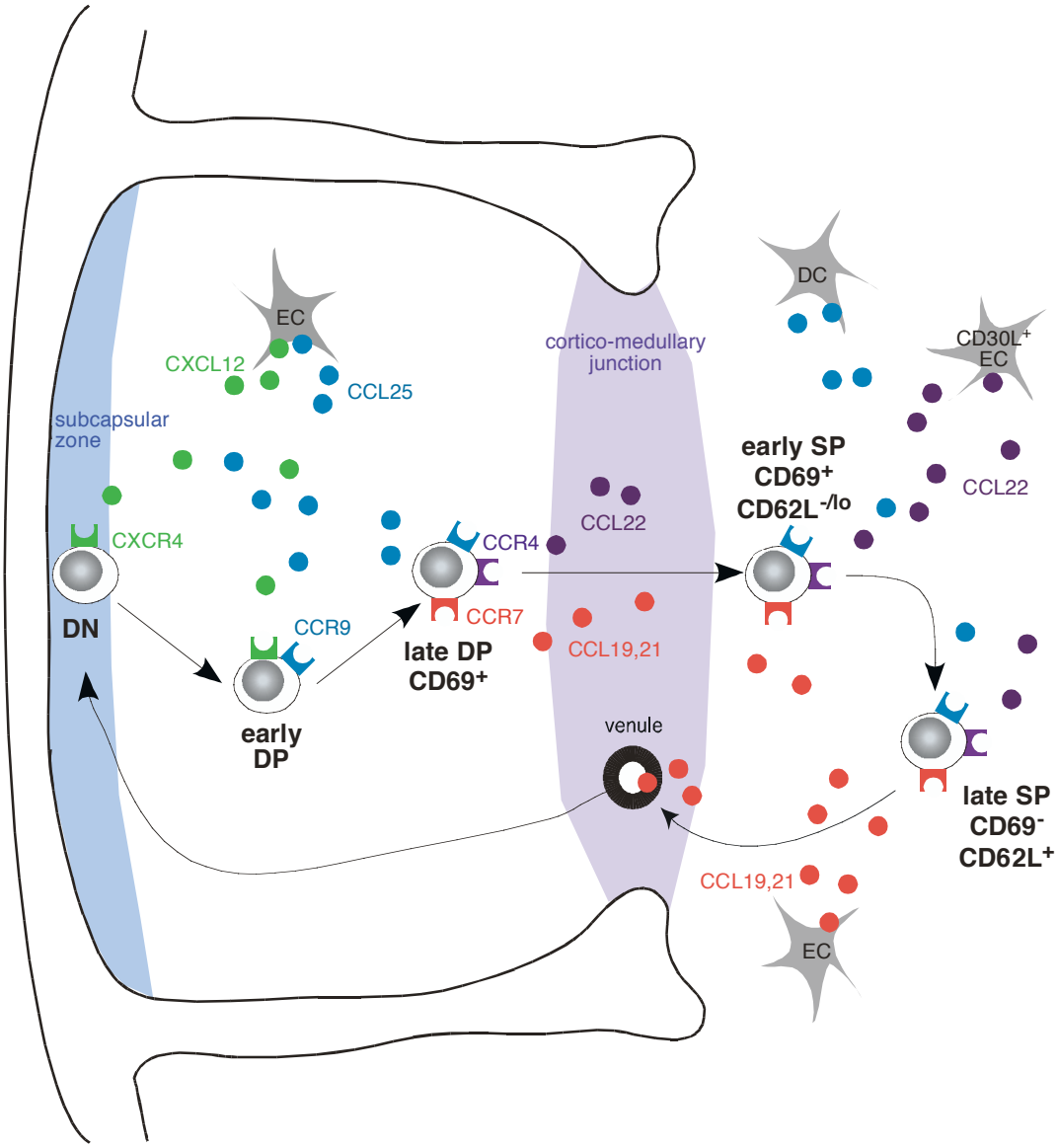

Figure 2. Chemokine and chemokine receptor expression in the thymus.
Shown are the major developmental stages of T precursors and their general location within the thymus. The organ is divided into the outer cortex (left) and inner medulla (right); the boundary zone is indicated by light purple, and the outermost border of the cortex, called the subcapsular zone, is represented in light blue. The chemokine receptors and their cognate ligands are represented in matched colors: green CXCR4/CXCL12, blue CCR9/CCL25, purple CCR4/CCL22, red CCR7/CCL19/CCL21. The chemokines are expressed either by stromal cells of epithelial origin (EC) or by dendritic cells (DC). Arrows indicate the typical migratory path of a developing thymocyte: pregenitors enter from the blood stream via venules in the cortico-medullary junction and traverse to cortex to the subcapsular zone. From the subcapsular zone, cells then migrate back across the cortex, through the medulla and then out via a venule (which also expresses CCL19).

activation of Erk and Ca^{++} signals, and treatment with PMA and Ionomycin is sufficient to induce CCR7 expression in DP thymocytes (97, 98, 102, 103). Since the CCR4 ligand CCL22 (MDC) is produced exclusively by epithelial cells in the medulla and at the CMJ, it is tempting to speculate that CCR4 mediates thymocyte migration into the medulla during positive selection (104). Furthermore, $CCL22^{+}$ thymic epithelial cells are predominantly $CD30L^{+}$ (102), suggesting that CCL22 may specifically recruit thymocytes to stromal cells that can induce negative selection. However there is little experimental evidence to support this hypothesis since negative selection is normal in $CD30^{-/-}$ mice (105). Although $CCR4^{-/-}$ mice have been generated, the thymic phenotype and negative selection have not been studied in these animals (106).

As described above, there are three current theories to explain thymic exit: 2 active mechanisms, fugetaxis and peripheral chemoattraction, and one passive mechanism, loss of thymic retention. 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_i-specific inhibitor, in thymocytes (107). In the search for the responsible receptor, attention has recently turned to the chemokine receptor CCR7 and the GPCR $S1P_1$.

SP thymocytes express the chemokine receptor CCR7 following the completion of positive selection and migrate *in vitro* to both of its ligands, CCL19 (ELC/MIP3ß) and CCL21 (SLC/6-Ckine/Exodus-2) (98). Furthermore, CCL19 is expressed in the structures by which mature cells leave the thymus, making CCR7 an attractive candidate for active chemoattraction mechanisms for thymic exit (102). However thymocyte emigration is unaffected in CCR7-deficient mice and in *plt*/*plt* mice, which lack lymphoid expression of both CCL19 and CCL21 (108-110). In fact, emigration is almost the only thymocyte movement that is not affected by loss of CCR7 signals. CCR7 is expressed briefly by early thymocytes during the DN1 to DN2 transition, and $CCR7^{-/-}$

DN2 cells ($CD25^{+}44^{+}$) accumulate at the CMJ, reflecting an inability to traverse the cortex to the subcapsular zone (111). Some cells do manage to migrate properly and successfully develop to the DP and SP stages. However $CCR7^{-/-}$ and *plt*/*plt* SP thymocytes consequently accumulate in the cortex because of defective migration into the medulla (112). The medullas of $CCR7^{-/-}$ and *plt*/*plt* thymii contain approximately 50% of the volume of wild-type medullas and display a marked lack of SP cells, even when SP numbers are increased due to expression of the AND Tg TCR (112). Intriguingly, CCR7-null and *plt*/*plt* SP thymocytes are phenotypically normal. Tg TCR thymocytes also undergo successful negative selection in mutant animals, suggesting that medullar localization is dispensable for selection and SP differentiation (112).

Recent studies of the immune-suppressant drug FTY720 have indicated that sphingoside lipid signaling may play an important role in multiple stages of lymphocyte trafficking (113). *In vivo*, FTY720 is metabolized into a sphingosine-1-phosphate (S1P) analog capable of binding the receptors $S1P_1$ (edg1), $S1P_3$ (edg3), and $S1P_5$ (edg5). $S1P_1$ expression is initially induced following positive selection and is progressively upregulated during SP thymocyte maturation (114). A small percentage (2-4%) of late-stage ($CD62L^{hi}$) SP thymocytes migrate *in vitro* in response to S1P (114). However, the exact role of S1P in thymic egress remains unclear: Rosen *et al*. report inhibition of thymic egress by $S1P_1$ agonists (115), but thymocyte emigration is also impaired in mice lacking $S1P_1$ (114, 116).

1.5 Structure and function of Class I_A PI3Ks

Phosphatidylinositol 3-kinases (PI3Ks) catalyze the phosphorylation of the D3 position of inositol head groups, generating PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3. Three classes of PI3Ks

are defined in reference to substrate specificity as well as structure and regulation. Class I PI3Ks can phosphorylate PtdIns(4)P and PtdIns(4,5)P_2, although the latter appears to be the preferred substrate (117). Class I_A PI3Ks consist of catalytic subunits that interact with SH2 domain-bearing adaptor subunits, whereas the Class I_B PI3K p110γ utilizes a p101 adaptor subunit and is stimulated by G protein βγ subunits. While multiple Class I_A subunits have been implicated in T lymphocyte signaling, the p85α and p110α subunits are thought to largely mediate PI3K function in T cells.

All Class I_A adaptor subunits are characterized by two SH2 domains, one of which is located at the carboxyl-terminus (Fig. 3). The intervening "Inter-SH2" domain mediates association with the catalytic p110 subunit. The multiple PI3K adaptor subunits are splice isoforms of three genes (α, β, and γ). The p85adaptors (α and β) are distinguished from the other Class I_A adaptor subunits by a unique, longer amino-terminus containing a SH3 domain and two proline-rich domains. The p85 subunits also include a RhoGAP homology domain that may interact with small GTPases such as Rac, but whether this domain has GAP catalytic function remains uncertain. The Class I_B adaptor subunit shows no similarity to any known proteins or domains, but deletion studies have suggested that association with the p110γ subunit is mediated by large portions of both the amino- and carboxyl-termini (118).

The class I_A catalytic subunits are characterized by four distinct domains. The amino-terminus mediates association with the adaptor subunit, and the Ras-binding domain appears to be critical for activation (227). The kinase domain is located at the carboxyl terminus of p110α. The crystallographic structure of p110α has not yet been determined, but that of the related class I_B p110γ (42% sequence identity) has revealed that the kinase domain is ordered into a small N-

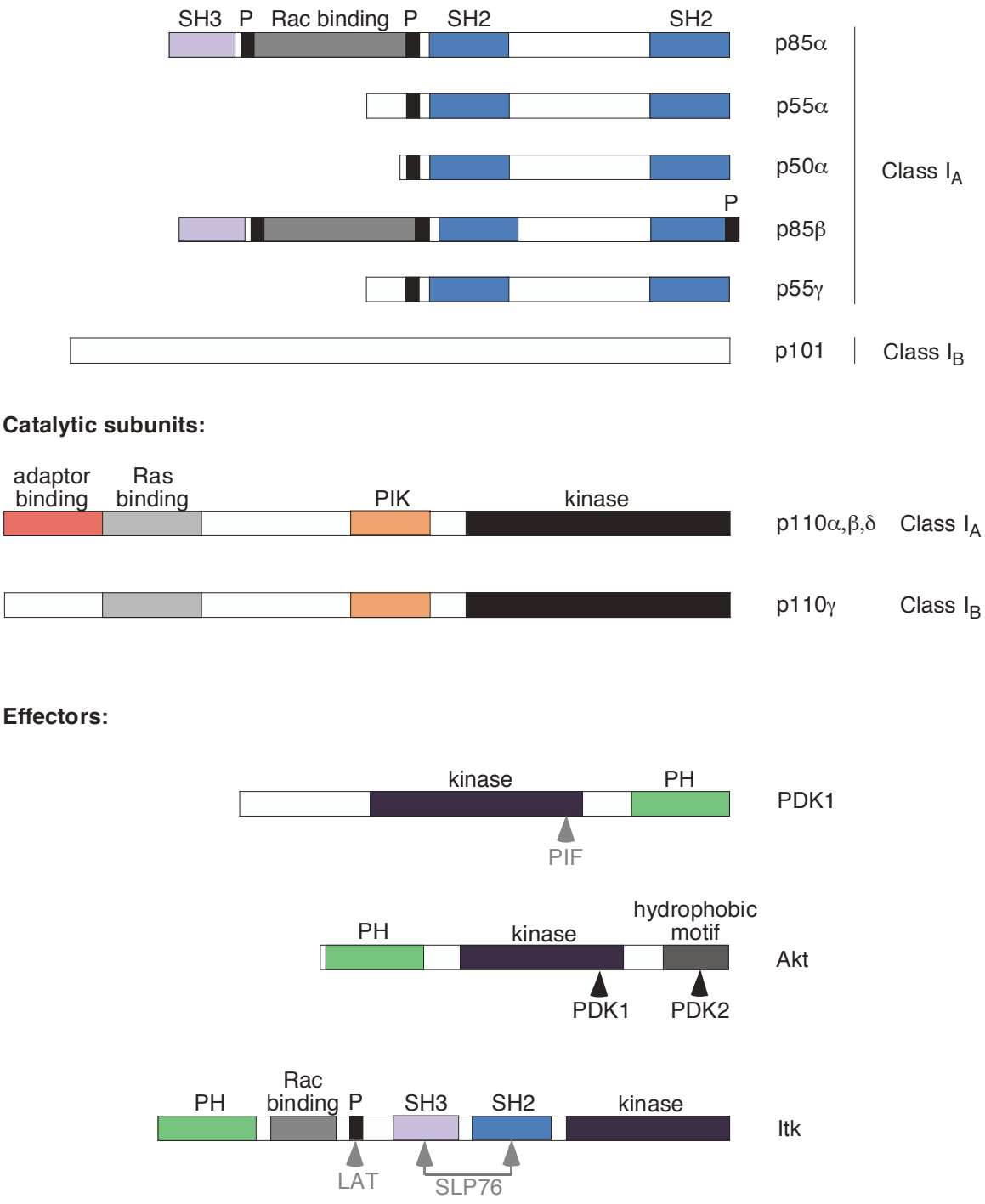

Figure 3. Structure of PI3K subunits and principal effectors
Black arrows indicate phosphorylation; grey arrows represent interaction partners; P = proline rich region.

terminal lobe and a larger C-terminal lobe that superimpose with the tyrosine kinase domain of Src (119). The highly-conserved and PI3K-specific PIK domain consists of anti-parallel helices that form a central domain, a spine upon which the other domains are fastened. Class I_B p110γ is highly identical to the class I_B p110s with the notable exception of the amino terminus, which has been implicated in association with p101 (118).

According to the classic model of class I_A PI3K activation, p85 is recruited to phosphorylated YXXM motifs via its SH2 domains, thus translocating p110 to the cell membrane (Fig. 4). Alternatively, class I_B PI3Ks associate tightly with and are activated by $G_{\beta\gamma}$ subunits. Membrane translocation allows association with Ras, an interaction that likely activates the catalytic subunit, and subsequent phosphorylation of membrane PtdIns (227). PI3K metabolites serve as docking sites for multiple cytosolic proteins. Two domains can bind PtdIns: Pleckstrin homology (PH) domains can associate with multiple phosphorylated PtdIns whereas FYVE motifs selectively associate with PtdIns(3)P (120). PH domains have been identified in over 150 proteins thus far, but only those with a $KX_{7\text{-}13}R/KXR\dagger$ motif (where X is any amino acid and † represents a hydrophobic residue) can bind PtdIns specifically (121). These domains can associate with PtdIns(3,4)P_2 but bind with higher affinity to PtdIns(3,4,5)P_3, which is absent in resting cells: PI3K activity thus specifically recruits these PH domain-containing proteins to the vicinity of ligated receptors. Membrane translocation is a hallmark feature of PI3K effector activation.

PI3K function is antagonized by two phosphatases specific for PtdIns(3,4,5)P_3. SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP) converts PI3K products into PtdIns(3,4)P_2, whereas phosphatase and tensin homolog (PTEN) dephosphorylates the 3' position to regenerate PtdIns(4,5)P_3. The antagonistic effects of these two phosphatases are required for proper PI3K regulation, and PtdIns(3,4,5)P_3 accumulate in resting cells lacking either. This effect

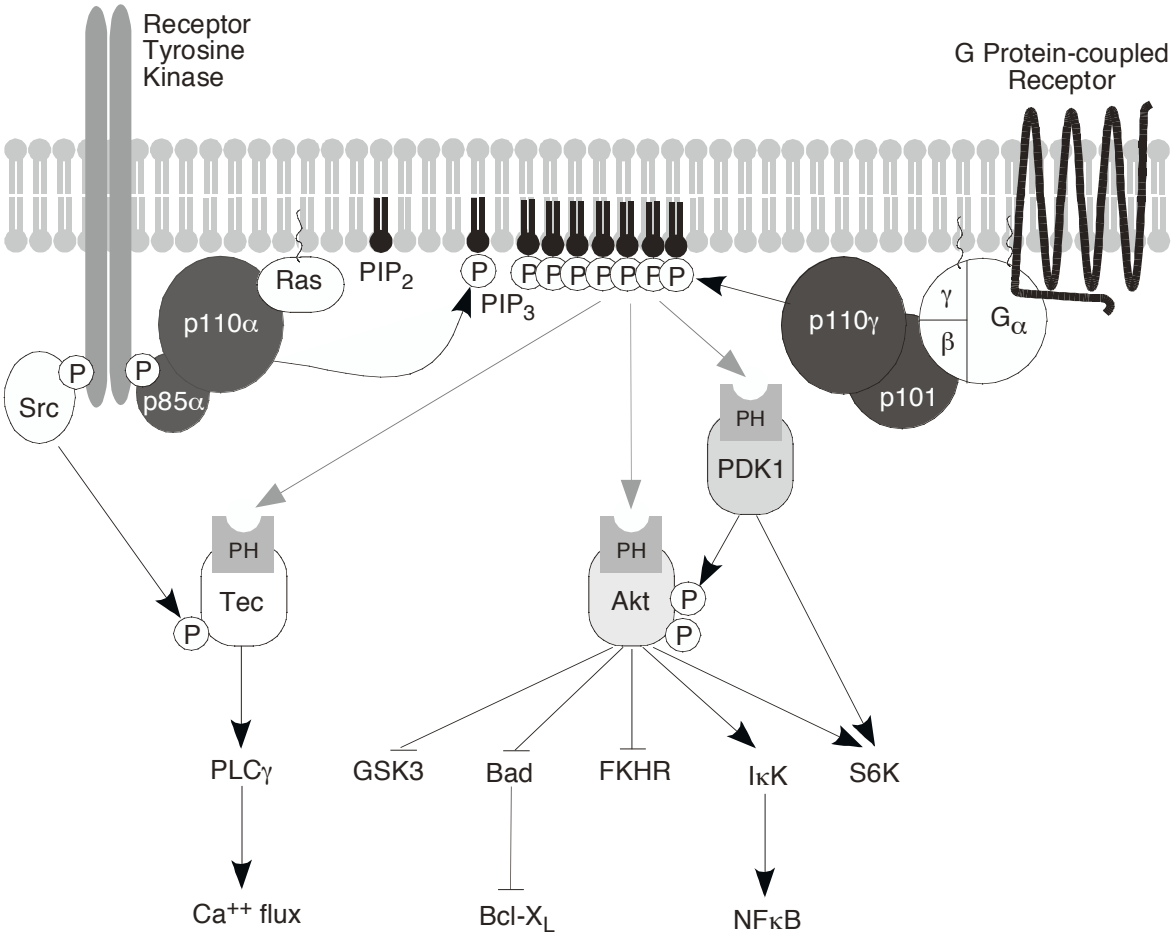

Figure 4. The prototypical model of PI3K signal transduction.
According to the standard model for PI3K activity, class I_A PI3K heterodimers are recruited to ligated receptor tyrosine kinases via the SH2 domains of the adaptor subunit (shown here as p85α). Translocation to the membrane allows the catalytic subunit to interact with Ras and to phosphorylate PtdIns(4,5)P_2 lipids in the inner cell membrane. PtdInsP_3 products are also generated by class I_B PI3K dimers that are associated with and activated by the bg subunits of G proteins coupled to serpentine receptors. The resultant local accumulation of PtdInsP_3 recruits PH domain-containing proteins. Once translocated to the membrane, Tec kinases are phosphorylated by the RTK-associated Src kinases and in turn activate PLCγ, thereby inducing Ca^{++} flux. PDK1 contributes to activation of Akt at PtdInsP_3-enriched domains. Akt phsphorylates a variety of substrates, ultimately regulating cell cycle, transcription, and survival (the latter via Bcl family members, FKHR, and NF-κB). Black arrows represent positive regulation, inverted T's represent inhibtion, grey arrows indicate physical association.

can be observed in the Jurkat T cell line, which lacks both PTEN and SHIP and exhibits constitutive membrane association of PI3K effectors; ectopic expression of SHIP1 in Jurkats is sufficient to increase cytosolic localization of the Akt PH domain (122, 123). There are two structurally-unrelated pharmacological inhibitors of PI3K, Wortmannin and Ly294002, which both bind directly to all p110 subunits.

I.6 PI3K effectors: Akt and other AGC kinases

The AGC family comprises a group of serine/threonine kinases that must be phosphorylated at two sites to become active, a threonine residue within the activation or "T" loop of the kinase domain and a C-terminal hydrophobic motif residue that may be a serine or threonine. Multiple AGC kinases are regulated by PI3K activity, but the best understood is Akt (PKB). Akt is recruited to PtdIns(3,4,5)P_3 via its amino-terminal pleckstrin-homology (PH) domain (124), and is activated at the membrane by phosphorylation of Thr308 and Ser473 (Fig. 3). Phosphorylation at Thr308 is mediated by the PI3K-dependent kinase PDK1, which is itself a PH domain-containing kinase and AGC family member (148). PI3K products may also contribute to Akt activation by relieving intra-molecular inhibition via altered conformation, allowing access to Thr308. This is suggested by the observation that PtdIns(3,4,5)P_3 is still required for Akt phosphorylation by a PDK1 mutant lacking the PH domain (ΔPH-PDK1) (125). The kinase responsible for phosphorylation at Ser473 has been designated as "PDK2" and its identity remains uncertain. Recently groups have discovered that PDK1 can physically associate with the PRK2 PIF (for PDK1-interacting fragment) domain, and this interaction induces PDK1-mediated phosphorylation of Akt Ser473 (126). On the other hand, Ser473 is still phosphorylated upon activation in PDK1$^{-/-}$ ES cells (127). Therefore any "PDK2" function exerted by PDK1 is

dispensable for PI3K-mediated Akt regulation. Following activation at the membrane, Akt rapidly moves to the cytosol and nucleus, where it phosphorylates a number of targets.

PI3K has been generally implicated in growth, cell cycle control, activation, and survival, and Akt appears to be a point at which many of these effects bifurcate. The first described Akt substrate was GSK3, originally characterized as a regulator of glycogen metabolism but since implicated in a wide variety of cellular processes (128). There are over 40 putative GSK3 substrates, including a large number of transcription factors (129). Many of these must still be verified experimentally, and certainly cell-type and cell-context specificity must be taken into account. This is especially evident in the varied roles of GSK3 in apoptosis. For example, transfection of PC12 and Rat1 cells with a catalytically active GSK3β induces apoptosis, whereas dominant negative forms promote survival (130). In contrast, mice deficient in GSK3β die during early fetal development in a manner consistent with TNF toxicity due to a loss of NF-κB function (131). In most pathways characterized so far, GSK3 functions as a repressor that is inactivated by Akt-mediated phosphorylation. The repressive capacity of GSK3 can be observed in its phosphorylation of NF-AT, promoting cytoplasmic sequestration (132). However, Akt has not been confirmed as an upstream regulator of this mechanism.

The best-described role of Akt is the regulation of cell survival, which is mediated via multiple downstream effectors in addition to GSK3. Akt directly phosphorylates the pro-apoptotic protein Bad *in vitro* and *in vivo* on Ser136 (133). Bad typically exerts an apoptotic function in cells by associating with and inhibiting anti-apoptotic Bcl-2 family members. Ser136 phosphorylation inactivates Bad, allowing the release (and derepression) of Bcl molecules such as Bcl-X_L (133). Expression of dominant-negative PI3K or Akt potentiates TNF-mediated killing of HeLa cells, which can be blocked by overexpression of Bcl-X_L (134). Furthermore, Ser136 of Bad is

essential for SCF-promoted cell survival of U2 OS (human osteosarcoma) cells (133).

Akt can also induce cell survival by the regulation of transcription. Genetic studies of insulin receptor/PI3K survival signaling in *C. elegans* identified the downstream gene DAF16, a member of the forkhead transcription factor family (135). There are three members of this family in mammals, FKHR, FKHRL1, and AFX, which upregulate expression of "death" genes such as FasL and Bim (136, 137). Akt-mediated phosphorylation at three sites inhibits forkhead transcriptional activity and directs nuclear export, whereupon the phosphorylated factor is bound by 14-3-3 proteins (136, 138).

PI3K may also positively regulate survival genes by inducing activation of NF-κB via Akt-mediated phosphorylation of IKKα. Transfection of 293 cells with either dominant-negative PI3K or kinase-dead Akt is sufficient to block NF-κB activation by TNF, and Akt physically associates with IKKα in fibroblasts following PDGF stimulation (139, 140). However the physiological importance of this putative pathway remains to be demonstrated. This mechanism is dependent upon IKK family member expression, since PI3K/Akt cannot regulate NF-κB in cell types expressing more IKKβ than IKKα (141). Furthermore, the IKKα phosphorylation site ascribed to Akt does not correspond to an Akt consensus site (121). Recently an alternative mechanism by which NF-kB can be regulated has emerged, e.g., via forkhead factors, but the requirement for upstream Akt activity remains to be demonstrated.

As mentioned above, PDK1 is an AGC family member as well and may exert PI3K functions independent of Akt activation. The mechanism by which PI3K regulates PDK1 has recently come into question. Some groups describe activation-dependent PDK1 membrane translocation (142, 143), whereas other studies identified a cellular fraction of PDK1 that is constitutively

associated with the plasma membrane (144). Since a membrane-targeted Akt (gagPKB) exerts constitutive activity *in vivo* (145), there must be some amount of membrane-associated PDK1 in resting cells. PDK1 expressed in bacteria is fully phosphorylated and active, indicating that PDK1 can autophosphorylate (likely in *trans*), and that kinase activity is activation-independent (146). It seems then that PDK1 function downstream of PI3K is instead regulated via its access to substrates, e.g., by Akt membrane localization.

The p70 S6 kinase (S6K) is yet another AGC kinase regulated by PI3K, apparently via the cooperation of Akt and PDK1. S6K T-loop phosphorylation is mediated by PDK1 *in vivo* and *in vitro* in a Wortmannin-resistant fashion, but maximal S6K activation requires hydrophobic motif phosphorylation (147). This phosphorylation appears to create a PDK1 docking site by mimicking the structure of the PIF domain from PRK2 (148). Hydrophobic motif Thr389 phosphorylation is sensitive to both Wortmannin and rapamycin treatment in multiple cell types (149), suggesting a role for Akt and mTOR in S6K regulation. Indeed, Akt directly phosphorylates mTOR *in vitro*, and the mTOR-binding protein RAFT1 phosphorylates S6K at Thr389 (150, 151). In this manner PI3K can regulate aspects of protein synthesis.

1.7 Other mediators of PI3K function

Most members of the Tec kinase family also contain PtdIns(3,4,5)P_3-specific PH domains. There are 5 Tec kinases expressed in the haematopoietic system of mammals. Of these five, four contain amino-terminal PH domains (Tec, Btk, Itk, and Bmx) that mediate PI3K-dependent membrane translocation, whereas the last (Rlk) contains an amino-terminal cysteine-rich domain that can be palmitoylated (152). With the exception of Rlk, Tec kinases are activated by the

coordinated function of both PI3K and tyrosine kinases in a surprisingly complicated fashion (153). At its simplest, PH domain-mediated membrane translocation recruits Tec kinases to receptor-associated tyrosine kinases that phosphorylate and activate the Tec family member. The critical role of PI3K in Itk regulation is pointed out by observation that deletion of the Itk PH domain prevents the Lck-mediated phosphorylation of Itk induced by TCR cross-linking (154). The Tec kinases also contain SH2, SH3, and proline-rich domains that mediate both intramolecular associations and associations with other proteins (Fig. 3). In T cells these domains participate in the assembly of a large "signalsome" complex: Zap70-mediated phosphorylation of adaptors LAT and SLP76 allows the formation of a multimeric complex associated with the membrane (see Fig. 1) (155-157). PLCγ is likewise recruited to the membrane via a PtdIns(3,4,5)P_3-specific PH domain and associates with the signalsome complex there (19, 158, 159). Tec kinases subsequently phosphorylate and activate PLCγ, thereby inducing DAG and Ca^{++} responses (160).

Tec kinases may also exert other, less-understood functions downstream of PI3K. Studies of a *D. melanogaster* Tec homolog have revealed a function in cytoskeletal organization, and Btk, Itk and Tec were each shown to bind to WASP, an actin-organizing molecule (161, 162). Tec kinases may also regulate transcription by direct interaction with transcription factors, since nuclear translocation upon activation has been described for both Btk and Itk as well as the PI3K-independent Rlk (152, 163, 164). One group showed that Btk directly phosphorylates STAT5 in a JAK3-independent manner both *in vitro* and *in vivo* (165). Furthermore, ectopic wild-type but not kinase-inactive forms of human Btk could rescue STAT5A phosphorylation in $Btk^{-/-}$ chicken B cells (165).

PtdIns(3,4,5)P_3-specific PH domains are also found in subgroups of GTP/GDP exchange factors

(GEFs), including those specific for the Rho family GTPases and the ARF-GTPases. In particular, many groups have tried to determine the relationship between the PI3K and the RhoGEF Vav. Vav contains a PH domain, and an early model described PtdIns(3,4,5)P_3-dependent Vav activation (166). This finding has since been fiercely challenged, however, and structural studies of PH domains suggest that Vav should not bind PtdIns(3,4,5)P_3 (167, 168). In conflicting experimental reports, TCR-induced phosphorylation of Vav1 in murine primary T cells is sensitive to Ly294002, but a Vav1 mutant with PH domain deletions is phosphorylated normally in Jurkat T cells (169).

P-Rex1 is another GEF that has attracted attention in the context of GPCR-dependent PI3K signaling. The Rac GEF activity of P-Rex1 isolated from neutrophil extracts was found to be PtdIns(3,4,5)P_3-dependent (170). P-Rex1 can associate directly with the $G_{\beta\gamma}$ subunit and is activated synergistically by PtdIns(3,4,5)P_3 and $G_{\beta\gamma}$ *in vitro* and *in vivo* (170). P-Rex1 does contain a PH domain but it is not very similar to domains with known PtdIns(3,4,5)P_3 specificity. Intriguingly, like p110γ, P-Rex1 exhibits significant basal association with the cell membrane, and it is therefore hypothesized to function as a "coincidence" factor that mediates the extremely rapid (as fast as 10 seconds) activation of Rac following GPCR stimulation (170). It has also been suggested that P-Rex1 participates in a feedback loop regulating the activity of multiple PI3Ks in chemotactic responses. According to this model, PI3Kγ and $G_{\beta\gamma}$ cooperate to initiate actin remodeling via P-Rex1 and Rac activation. Rac will subsequently bind to class I_A adaptor subunits to stimulate sustained PI3K activity. Recently the related P-Rex2 was identified, indicating possible cell-type specificity of P-Rex usage (171).

1.8 PI3K activation in T lymphocytes

PI3K is responsive to a variety of stimuli in T cells including stimulatory signals (CD3, CD28, ICOS), inhibitory receptors (CTLA-4), and growth factors and cytokines (IL-2, -4, -7). PI3K binds directly to CD28, ICOS, and CTLA-4 via the YXXM motif located in the cytoplasmic regions of each receptor. Phosphorylation of the tyrosine residue in this motif, possibly by the Src kinases Lck and Fyn, allows association with the SH2 domains of p85 (172). Other molecules known to activate PI3K, such as the TCR, CD4 and IL-2R, lack such sequences. Association of these receptors with PI3K may instead be mediated by other motifs such as YVXV and YLVL, as has been shown with hepatocyte growth factor receptor and EPOR in other cell types (173). Following TCR cross-linking, PI3K has been shown to associate with the TCRζ and ε chains via co-immunoprecipitation (173); however, such biochemical techniques have proven unreliable in T cells due to the plethora of adaptor molecules which form large complexes of multiple proteins. Many of the complex components do not interact directly or specifically. For example, TCR-interacting molecule (TRIM) contains a YXXM motif that is phosphorylated upon activation and recruits p85α to the TCR (174). Furthermore, many signaling molecules have been found to be constitutively associated with glycolipid-enriched domains (GEDs or rafts) independently of known protein-protein interactions (175). Hence association of PI3K with the TCR and other receptors may be direct or mediated via adaptor proteins (e.g., TRIM), rafts, or Src family members.

PI3K is activated extremely rapidly in T cells following TCR cross-linking, even preceding increases in intracellular Ca^{++} (176). Furthermore, studies in animals expressing GFP fused to the PH domain of Akt demonstrate that PI3K activity is maintained many hours following TCR/MHC interaction (177). This sustained activity is required for some TCR-induced

processes, since addition of Ly294002 within the first 9 hours is sufficient to completely block proliferation (177). A role in TCR-induced PI3K-mediated proliferation has been further supported by the study of mice with genetic modifications of PI3K signaling. For example, T cells of $p110\delta^{D910A}$ animals, in which a kinase-inactive form of p110δ is knocked into the endogenous allele, exhibit reduced proliferation in response to TCR cross-linking, only 50% that of wild-type cells (178). This diminished response correlates with a nearly complete loss of Akt phosphorylation, decreases in Ca^{++} flux, and reduced IL-2 production. Interestingly, defective T cell responses in $p110\gamma^{-/-}$ animals suggest that even this GPCR-linked PI3K may play a role in T cell activation (175). Conversely, PI3K signaling can be potentiated by deletion of PTEN: compared to wild-type, PTEN-deficient T cells are hyperresponsive in mixed lymphocyte reactions, produce higher amounts of cytokines, and demonstrate increased TCR-induced Akt kinase activity (82).

The studies of knock-out and transgenic mice also provide the best indication of which PI3K effectors are important in TCR signaling. Two strains of mice have been generated with ligand-independent Akt activity via constitutive membrane targeting. The gagPKB transgene resembles the oncogenic v-Akt, in which Akt is fused to an N-terminal gag sequence that is myristoylated (179). Antigen-dependent proliferation is markedly enhanced by gagPKB expression, as is antigen-independent viability *in vitro*. Independently another group fused the Lck myristoylation signal sequence to human Akt to generate a myrPKB mutant (180). MyrPKB expression improves proliferative responses of thymocytes to anti-CD3 cross-linking as well as increases and sustains GSK3 phosphorylation (180). PI3K-dependent regulation of GSK3 downstream of the TCR is also indicated by reduced proliferation and IL-2 production observed in T cells expressing a catalytically active form of GSK3β (181). In the same report, GSK3 function in T cells is ascribed to regulation of NF-AT, as nuclear localization of this transcription factor is increased by

treatment with lithium, an inhibitor of GSK3 (181).

Three Tec family members are expressed in T cells, all of which are phosphorylated following TCR cross-linking: Tec, Itk, and Rlk. Studies of mice deficient for members of this family reveal compensatory mechanisms that suggest usage by the TCR is largely indiscriminate. Single knock-out animals exhibit graded disruptions of T cell function, and significant defects are only observed in double knock-out mice. IL-2 production by $Rlk^{-/-}$ splenocytes stimulated with anti-CD3 antibodies is half that of wild-type cells; $Itk^{-/-}$ splenocytes produce even less, and $Rlk^{-/-}Itk^{-/-}$ T cells produce negligible quantities (182). Singly-deficient T cells display a surprising ability to proliferate in response to TCR cross-linking, but doubly-deficient cells are absolutely unable to proliferate, even in mixed lymphocyte reactions (182). These defects were attributed to severely impaired Ca^{++} flux responses consequent to delayed activation of PLCγ (182).

Most of the initial work on PI3K in T cells was based on the hypothesis that it was the principal signal contributed by CD28-mediated costimulation. Over the years, the literature on this subject has proven frustratingly unclear. Part of the difficulty lies in the experimental approaches of early studies, which depended either upon the pharmacological inhibitors of PI3K, the specificity of which can never be certain, and/or upon use of the Jurkat T cell line, which has defects in PtdIns(3,4,5)P_3 regulation due to deficiencies in SHIP and PTEN. To date the only genetic modification of PI3K that modifies CD28 function in primary T cells is the $p110\delta^{D910A}$ mutant. Splenocytes from these animals are surprisingly competent to proliferate and produce IL2 in response to co-ligation of CD3 and CD28 (178). Glycolipid-enriched raft recruitment to sites of interaction with anti-CD3 + anti-CD28-coated beads is significantly impaired, however (178). Early studies of lipid raft involvement in TCR signaling suggested that raft mobilization is the principal contribution of CD28-induced costimulation (183); PI3K may mediate this function of

CD28 costimulation.

There is some evidence that PI3K can play a negative regulatory role in TCR function. Expression of a constitutively active PI3K chimera, rCD2p110, completely abrogates NF-AT activity in Jurkat and EL4 cells induced by CD3 cross-linking but not by phorbol myristate acetate (PMA) plus Ionomycin (57). This effect is not mediated by Akt or Rac, as expression of constitutively active Akt or V12Rac does not recapitulate the inhibition (57). Conversely, expression of a dominant negative PI3K mutant, Δp85, induces a small increase in basal NF-AT activity and synergistically enhances TCR induction of NF-AT (57). NF-AT activation by the TCR is transient, probably due to a negative-feedback mechanism that has not been characterized. PI3K effectors may be part of this regulation and mediate termination of NF-AT activity. This function of PI3K could be induced by CTLA-4, to which PI3K binds via a YVLM motif in the cytoplasmic domain (173, 16).

PI3K activation is a hallmark of chemokine signaling in T cells as well as other cell types, as mentioned above. CXCL12 treatment induces the rapid and transient accumulation of PtdIns(3,4,5)P_3 in Jurkat T cells: PI3K products are detectable within 15 seconds and return to basal levels by 5 minutes following stimulation (184). Furthermore, SHIP-deficient thymocytes display enhanced chemotactic responses to CXCL12 (185). To date, chemokine-induced Akt phosphorylation has been reported for almost all T cell chemokines, including the thymic chemokines CXCL12, CCL19, CCL22, and CCL25 (184, 186, 187). Chemokine-induced Akt phosphorylation is biphasic, apparently due to the coordinated regulation of both class I_A and I_B PI3Ks (184). PI3Kγ was first implicated in CXCR4 signaling by the observed pertussis toxin-sensitivity of Akt phosphorylation (184). Subsequent studies in Jurkat cells using a Tet-inducible system demonstrated that CXCL12-mediated Akt phosphorylation and chemotaxis are inhibited

but not blocked by expression of dominant negative PI3Ks, either Δp85 or kinase-dead p110γ (188). Notably, the addition of Ly294002 further inhibits chemotaxis of mutant-expressing cells, suggesting that a complete block of chemotactic responses requires the complete inhibition of both class I_A and class I_B PI3Ks. The role of p85/p110 dimers in chemokine function is deduced from the lipid kinase activity detected in immunoprecipitates from CXCL12-stimulated T cells, but the kinetics of p85/p110 activation are much slower than that of PtdIns(3,4,5)P_3 accumulation (184, 189). PtdIns(3,4,5)P_3 accumulation instead correlates with the rapid activation of PI3Kγ observed by 30 seconds following CXCL12 treatment (184). By contrast, p85-associated kinase activity is not maximal until 20-30 minutes, which instead matches the maximal cell polarization observed in response to CXCL12 (189). This suggests that class I_A PI3Ks are required for functions other than lipid kinase activity, but CXCL12-stimulated Jurkat cells do exhibit sustained Akt phosphorylation (up to 90 minutes at high concentrations of CXCL12) that may be p85/p110-mediated (184). Although class I_A PI3Ks are almost certainly required for the signaling of most chemokine receptors, exactly how these heterodimers contribute to chemotactic responses remains unclear.

1.9 PI3K and its effectors in lymphocyte development

Multiple studies have described a critical role for PI3K and its downstream effectors in B lymphocyte development. The mutant *xid*, a murine model for human immunodeficiencies, has been mapped to mutations in the PH domain of the Tec family member Btk. Specific disruption of p85α imposes a block on B cell development that results in a phenotype reminiscent of that of $Btk^{-/-}$ mice: reduced numbers of mature B lymphocytes and a block at the pro-B to pre-B transition (190). This would suggest that PI3K plays a crucial role downstream of the pre-BCR.

On the other hand, the role of PI3K in T lymphocyte has proven more elusive. Unlike B cell development, thymocytes are unaffected in p85$\alpha^{-/-}$ mice (190). The lack of a defect in thymocytes may be explained by the presence of p55α and p50α isoforms that are left unchanged and appear to compensate for the p85α deficiency in the T cell lineage; PI3K activity in p85$\alpha^{-/-}$ T cells is 30% of normal, versus only 5% in B cells (190). Disruption of all three p85a isoforms causes neonatal lethality, but p85α55α50$\alpha^{-/-}$ ES cell reconstitution of RAG2$^{-/-}$ mice can generate normal thymii as assessed by FACS analyses (191). However, again PI3K activity js not completely abolished in p85α55α50$\alpha^{-/-}$ thymocytes but rather reduced by 70%, and p85β expression appeares to be upregulated (191). p85α55α50$\alpha^{-/-}$ peripheral T cells are fully competent to proliferate in response to IL-2 treatment or to TCR cross-linking alone or with CD28, and these responses are strongly inhibited by pretreatment with Ly294002 (191).

Whereas disruption of p85α isoforms has severe effects in knockout mice including lethality, deletion of the p85β isoforms does not affect the viability of animals. Thymocyte development appears grossly normal in these mice, according to flow cytometry analyses (192). Disruption of p110α results in early embryonic lethality between E9.5 and E10.5 characterized by a profound proliferative defect (193). p110β-deficient embryos also die by E10.5, but p110$\gamma^{-/-}$ and p110$\delta^{-/-}$ embryos are viable (175, 194-6). Intriguingly, p110δ^{D910A} has been construed as a dominant negative allele since T cells from these animals exhibit greater defects than p110$\delta^{-/-}$ T cells (178, 196). Neither modification affects thymocyte development, however, suggesting that this catalytic subunit is not utilized in the thymus (178, 196).

The most popular dominant negative PI3K mutant is a p85 protein lacking the inter-SH2 domain that mediates association with p110, Δp85. Expression of Δp85 in FTOC inhibits proliferation

and/or survival of thymocyte: the number of transgene-expressing thymocytes at initiation is roughly one third that of thymocytes transfected with an empty vector (197). After 18 days in FTOC, six-fold fewer thymocytes are transgene positive, indicating some possible role of PI3K in early thymocyte development. Despite reduced numbers, both TCRαβ and TCRγδ cells are observed in the transgenic FTOCs as well as normal percentages of DP and SP thymocytes (197). The role of PI3K in DN thymocytes may be downstream of IL-7R. This is suggested since DN2 and DN3 survival is significantly impaired in IL7Rα$^{-/-}$ mice expressing a mutant IL-7Rα transgene lacking the intracellular domain that mediates PI3K activation (198). On the other hand, p110γ$^{-/-}$ mice have higher percentages and numbers of DN thymocytes (81). These DN cells are primarily small, pre-β-selection cells and include almost no large, post-β-selection cells, indicating that p110γ deficiency disrupts preTCR rather than IL-7R signals. DN numbers and the large DN3 fraction are restored to normal in p110γ$^{-/-}$ by the transgenic expression of p65^{PI3K}, an activating form of p85α that slightly increases basal PtdIns(3,4,5)P_3 levels and enhances TCR-induced PI3K activity (81, 199).

Although PI3K can regulate mature T cell survival, the importance of these mechanisms in DP thymocytes remains unclear. p110γ$^{-/-}$ mice were generated independently by two groups, and both report slight DP decreases. This may be due to impaired β-selection, as observed by Rodriguez-Borlado *et al.*, or it may be due to the increased susceptibility to GPCR-induced apoptosis observed by Sasaki *et al.* (81, 175). *In vitro* DP survival is enhanced for both PTEN-deficient and gagPKB thymocytes but is largely unaffected in myrPKB mice (82, 179, 180). Intriguingly, Dexamethasone-induced apoptosis of PTEN-deficient and p110γ$^{-/-}$ thymocytes is the same as wild-type (82, 175). DP numbers are also normal in mice from both Akt transgenic lines, indicating that the observed changes *in vitro* likely do not reflect *in vivo* thymocyte survival

mechanisms (179, 180).

PI3K does appear to play a role in positive selection, as neonatal $p65^{PI3K}$ mice have twice as many $CD3^{hi}$ thymocytes as wild-type animals (81). Whether this effect is mediated by Akt is unclear. Whereas Jones *et al*. observe no change in the positive selection of Tg TCR thymocytes in gagPKB mice, myrPKB expression improves development of such thymocytes (179, 180). Positive selection is clearly inhibited by the loss of Tec family members, however. Like the graded reduction of TCR signals in these animals, the degree of inhibition correlates with the identity of the deleted kinase: $Rlk^{-/-}Itk^{-/-}$ > $Itk^{-/-}$ > $Rlk^{-/-}$(200). To date the positive selection of five Tg TCR, both class I- (HY, OT-1) and class II-restricted (AND, 2B4, 5CC7), have been studied in these mice. Positive selection is always impaired by the loss of Tec kinases (85-87). The severity of the defect also correlates with the avidity of the interaction between TCR and its selecting peptide. For example, the selection of AND Tg TCR thymocytes was severely compromised by Itk deficiency in the weakly-selecting $H\text{-}2^{b}$ background, whereas selection was only modestly inhibited in the highly selecting $H\text{-}2^{k}$ background, and an intermediate effect was observed in $H\text{-}2^{k/b}$ animals (87). It should be noted, however, that the positive selection that remains in these animals could be mediated by endogenously-rearranged TCRα chains since all mice were Rag-competent. Positive selection appears to be more severely compromised in $Rag^{-/-}$ $Itk^{-/-}$ mice than $Rag^{+/+}$ $Itk^{-/-}$ (200). The role of Tec kinases in positive selection has also been described in mice expressing an Rlk transgene. In fact, Rlk over-expression can almost completely restore positive selection of HY $Itk^{-/-}$ and AND $Itk^{-/-}$ thymocytes (16).

The effects on positive selection mediated by PI3K and Akt mutants exhibit a selective involvement in the CD4 lineage. CD8 SP numbers are unaffected when myrPKB is expressed in OT-1 Tg TCR mice (180). Surprisingly, OT-1 myrPKB animals exhibit enhanced development

of CD4 SP thymocytes that are phenotypically mature and the spleens from these animals contain more Tg TCR $V_\alpha 2^{hi}$ CD4 T cells than OT-1 littermates. The expression of $p65^{PI3K}$ similarly redirects development of thymocytes expressing the class I-restricted F5 Tg TCR into the CD8 lineage (81). p110γ deficiency inhibits the differentiation of CD4 SP while potentiating CD8 SP development in mice expressing the class II-restricted TCR 5CC7 (81). These observations are in stark contrast to those of Rlk- and Itk-deficient animals, in which lineage commitment is unchanged (87).

Tec kinase deficiencies also reduce the efficiency of negative selection according to several experimental models of this process. For example, significantly increased numbers of DP and CD8 SP thymocytes are observed in HY Tg TCR mice doubly-deficient for Rlk and Itk, and these cells express higher levels of CD8 than those in wild-type HY animals (86). As observed for positive selection, the inhibition of negative selection is partly dependent upon the affinity of the TCR for the interacting ligand. Superantigen-mediated deletion of DP thymocytes is more severely impaired for the low-affinity 2B4 Tg TCR than for the high-affinity AND Tg TCR in $Itk^{-/-}$ mice (200). In view of Tec kinase-induced PLCγ function, these results agree with the proposed role of Ca^{++}-regulated Nur77 in negative selection.

2. Generation of a model system to study the molecular biology of PI3K and its role in thymocyte development

2.1 $p110_{ABD}$ gain-of-function transgenic mice

Since PI3K isoform knockout animals have yielded either no effect on thymocyte development or early embryonic lethality, we have used an alternative approach to analyze the role of PI3K at the transition between immature DP and mature SP thymocytes. Transgenic expression of a gain-of-function mutant ($p110_{ABD}$) 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 antigen presenting cells (201-203). We generated four lines of mice that express the $p110_{ABD}$ transgene, consisting of amino acids 1-108 of the bovine Class I_A catalytic subunit p110α. This adaptor-binding domain (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. 5A) (204), and the 9E10 Myc epitope is included at the N-terminus for antibody recognition.

We can easily co-immunoprecipitate p85α with $p110_{ABD}$ via recognition of the Myc tag by the 9E10 antibody (Fig. 5D), but we observe decreased levels of endogenous full-length p110α (Fig. 5C). We originally expected $p110_{ABD}$ to exert a dominant negative effect over PI3K function: a similar truncation mutant containing the first 126 amino acids (base pairs 1-379) was reported to inhibit EGF-mediated expression of a reporter plasmid driven by the TPA response element (205). We were therefore surprised to observe constitutive activity in $p110_{ABD}$ thymocytes when we assay Akt kinase activity as a surrogate readout (Fig. 6A). We believe that $p110_{ABD}$ induces PI3K activity by disrupting the normal p85-mediated regulation of endogenous p110 subunits.

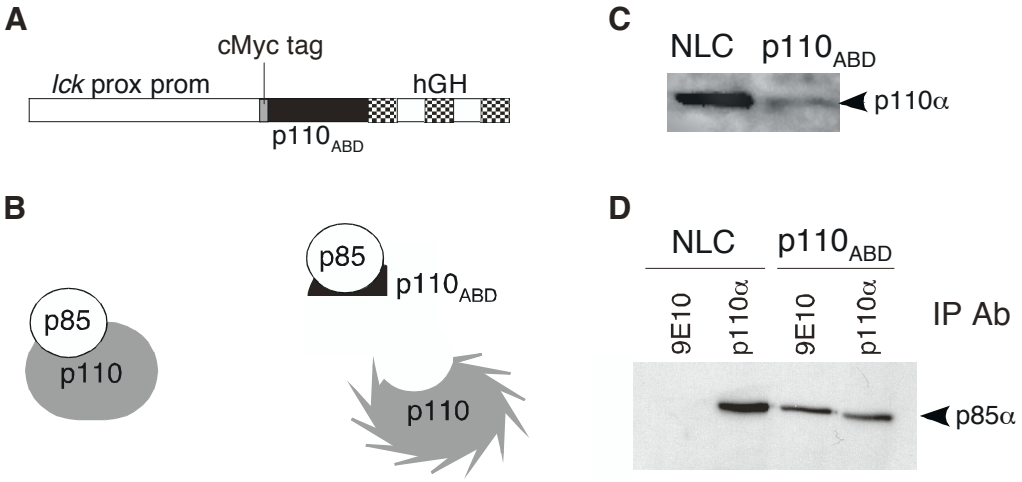

Figure 5. The $p110_{ABD}$ transgene product sequesters endogenous p85.
A. Diagram of the transgenic contruct utilized to generate $p110_{ABD}$ mice. The *lck* proximal promoter drives transgene expression in thymocytes only. The cMyc tag sequence recognized by the 9E10 antibody was cloned at the N-terminus of the transgene, and hGH repeats allow genotyping by PCR and dot blot.
B. Model of $p110_{ABD}$ function. In normal cells the adaptor p85 is bound to wild-type p110 kinase, increasing its thermal stability but inhibiting kinase activity. In thymocytes from $p110_{ABD}$ transgenic mice, the adaptor binding domain (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 wild-type, full-length p110α in thymocytes from NLC and $p110_{ABD}$ transgenic mice.
D. p85α can be co-immunoprecipitated with $p110_{ABD}$ via the N-terminal cMyc tag of the transgenic protein by the 9E10 antibody. Immunoprecipitations were performed upon 3 x 10^7 thymocytes per lane.

The mechanism by which p85 regulates catalytic subunits has proven to be far more complex than originally suspected. In the classical model described above in Section 1.5, adaptor subunits function merely as scaffolding proteins that regulate the subcellular location of catalytic subunits. That this is an oversimplification was first suggested by the observation that *in vitro* kinase activity could be potentiated merely by addition of peptides containing phosphorylated YXXM motifs (206). When p110α is expressed in insect cells in the absence of p85α, significant lipid kinase activity is observed (207). Co-expression with p85α increases the amount of p110α isolated from cell extracts but dramatically decreases kinase activity (207). Similar results are observed with tagged forms of p110α in HEK 293T cells. A C-terminal Myc-tagged p110α monomer exhibits a half-life of only 1 hour that is increased 5-fold by p85α co-expression (207). Intriguingly, the addition of large, N-terminal GST tags could substitute for p85α co-expression with regards to thermal stability (207). Furthermore, kinase activity is decreased 50% by p85α co-expression when GST-p110α is expressed in cells at 30°C, a temperature at which monomeric GST-p110α is far more stable (207). Deletion analysis of p85α has indicated that the N-terminal SH2 domain mediates both inhibition of lipid kinase activity and disinhibition by phosphopeptides (208). These results suggest a model in which association with p85 confers stability at 37°C but inhibits p110 lipid kinase activity. Activation-induced translocation of the dimer to phosphorylated sequences induces a conformational change that in turn activates lipid kinase activity.

These results suggest that $p110_{ABD}$-mediated sequestration of adaptor subunits allows endogenous p110α to exist as an unstable monomer. This model is depicted in Figure 5B. Monomeric p110 subunits have constitutive lipid kinase activity, and indeed we observe the constitutive activation of Akt, a well-known downstream effector of PI3K, in the thymocytes of mice expressing the

$p110_{ABD}$ transgene. As shown in Figure 6A by an *in vitro* kinase assay on Akt immunoprecipitates, unstimulated $p110_{ABD}$ thymocytes display the same maximal level of Akt activity that is induced in wild type (NLC, non-transgenic littermate control) thymocytes following 2 minutes of stimulation with plate-bound anti-CD3, suggesting that $p110_{ABD}$ transgene expression induces a physiological level of PI3K activity.

The observed decrease in Akt activity following CD3 cross-linking on the surface of $p110_{ABD}$ thymocytes is intriguing, but as yet unexplained. This suggests the activation of a TCR-dependent, PI3K-potentiated feedback mechanism for the regulation of Akt. Currently only two molecules are known to negatively regulate Akt, the lipid phosphatases PTEN and SHIP. Although both do so by depleting the cell membrane of PtdIns(3,4,5)P_3, most attention has focused on PTEN. PTEN is expressed constitutively in the thymus but almost nothing is known about how its activity is regulated. Preliminary studies point to internal PEST and PDZ domains and to PI3K itself. Whether it be PTEN or another mechanism dampening Akt activity in $p110_{ABD}$ thymocytes following stimulation, it must require non-PI3K-dependent signals to be activated.

2.2 Function of Akt effectors in $p110_{ABD}$ thymocytes

Various studies have shown that Akt can regulate cell survival through multiple effectors, particularly via regulation of Bad/Bcl-X_L dimerization (209, 210). Bad contains the consensus binding site for Akt and can be phosphorylated by Akt *in vitro* and *in vivo* (209). We examined Bad phosphorylation in $p110_{ABD}$ thymocytes by immunoblotting using an anti-Bad antibody to detect total unphosphorylated and size-shifted phosphorylated Bad as well as a phospho-Bad-

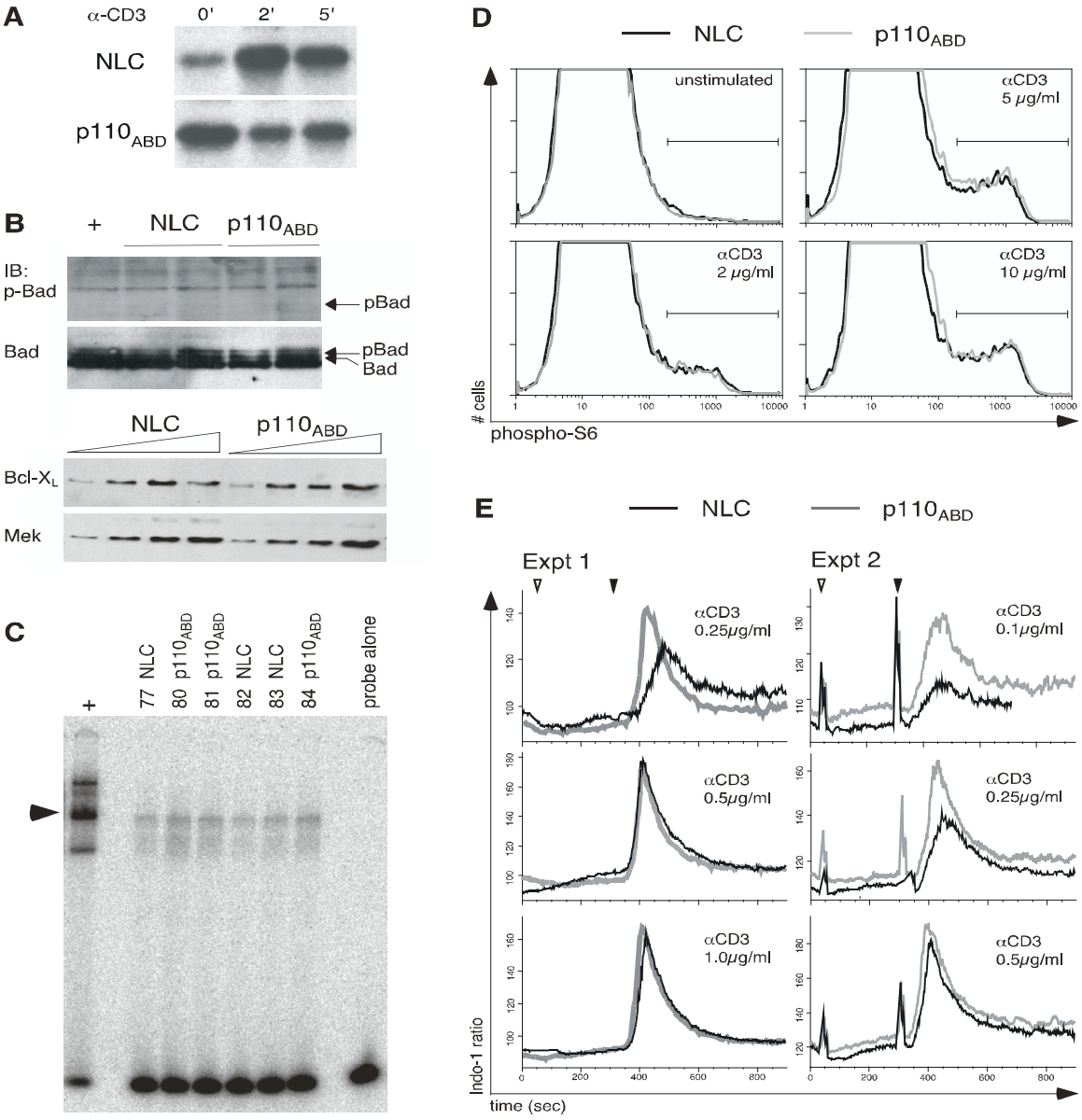

Figure 6. $p110_{ABD}$ expression activates Akt and potentiates Ca^{++} flux.

A. Constitutive Akt activity is observed in resting $p110_{ABD}$ thymocytes. Thymocytes were stimulated with 10μg/ml plate-bound anti-CD3 and in vitro kinase assays were performed with Akt immunoprecipitates.

B. Bad phosphorylation and Bcl-X_L protein levels are not increased by $p110_{ABD}$. Equal amounts of protein were loaded per well for the top blots, whereas samples were titrated for Bcl-X_L blotting. The Mek antibody was used to show equivalent loading between NLC and $p110_{ABD}$.

C. $p110_{ABD}$ does not induce increased NF-κB activity, as measured by the ability to gel-shift a radiolabeled hairpin target (shifted complex is indicated by an arrowhead).

D. S6K activation by CD3 crosslinking is not enhanced by $p110_{ABD}$ expression. Cells were stimulated for 10 minutes on antibody-coated plates.

E. $p110_{ABD}$ DP thymocytes exhibit maximal Ca^{++} influx at lower concentrations of crosslinking antibody than NLC DP cells. The open arrow represents the addition of 2C11 anti-CD3 at the indicated concentration, and the black arrow indicates when the secondary crosslinker was added (Goat-anti-Hamster, 10μg/ml).

specific antibody. We observed no difference in Bad phosphorylation in $p110_{ABD}$ thymocytes compared to NLC (Fig. 6B).

Akt has been reported to regulate NF-κB function via direct IKK activation in 293 cells *in vivo* (211). Additionally, an alternative mechanism for Akt-mediated IKK activation via Cot has recently been described. This MAPKKK associates with Akt in 293T cells and can be directly phosphorylated by Akt *in vitro* (212). Mutation of the serine phosphorylated by Akt abrogates Cot-mediated activation of IKK *in vitro* and *in vivo* in 293T cells. Importantly, this mutation also abrogates Cot-induced NF-κB activity in Jurkat T cells (212). Since co-expression of constitutively active myrAkt with Cot can activate NF-κB reporter gene expression in PTEN-competent T cells, we sought to determine if NF-κB is constitutively activated downstream of Akt in $p110_{ABD}$ thymocytes. However, when we measure NF-κB DNA-binding activity in thymocytes by EMSA, we find that $p110_{ABD}$-mediated constitutive activation of Akt is not sufficient to activate NF-κB in resting thymocytes (Fig 6C).

Our observations that $p110_{ABD}$ expression does not upregulate the activity of Bcl-X_L or NF-κB via Akt is in contrast to the description of these pathways in gagPKB mice (179). Although $p110_{ABD}$ induces constitutive Akt activity, Bcl-X_L levels are unaffected (Fig. 6B). By comparison, Bcl-X_L levels are dramatically increased in resting gagPKB thymocytes. The lack of $p110_{ABD}$-induced Bcl-X_L correlates with inability of $p110_{ABD}$ to mediate antigen-independent Bad phosphorylation, but Bad phosphorylation was not examined in gagPKB thymocytes. Bad phosphorylation in mature T cells is unaltered by gagPKB expression, however, suggesting that Akt can regulate activity of Bcl proteins through some other mechanism that remains obscure. Finally, it should be noted that the difference in Akt-mediated Bcl-X_L function in $p110_{ABD}$ and gagPKB thymocytes

correlates with different survival capabilities, which will be discussed below.

Similarly, NF-κB activity was not examined in gagPKB thymocytes but was described to be activated more efficiently in mature gagPKB T cells. Given the inexact conservation of TCR-induced signaling in thymocytes and T cells, there is a danger in extrapolating from one to the other. With this in mind, it is difficult to make direct comparisons between $p110_{ABD}$ thymocytes and gagPKB T cells. With regards to NF-κB regulation, however, we do have some comments. Firstly, we did not examine TCR-induced NF-κB activity, and it is thus possible that $p110_{ABD}$ may potentiate, rather than activate, NF-κB. But we do note that Akt activity is actually downregulated more rapidly following TCR stimulation in $p110_{ABD}$ thymocytes compared to NLC, which suggests that activation-dependent enhancement is unlikely. Secondly, to date there is little evidence to suggest that Akt regulates NF-κB in thymocytes *in vivo*. Expression cloning from a thymus cDNA library identified a novel regulator of NF-κB, called Card11 (18). It is likely that NF-κB activity in thymocytes is instead regulated via a pathway associated with this protein.

We also attempted to assess TCR-induced regulation of other putative Akt effectors, including GSK3, FKHR, and S6K, and any effect of $p110_{ABD}$ upon these. Our efforts were hampered in part by the very low levels of phosphorylation of these factors in thymocytes due to low CD3 surface expression on DP. We were unable to achieve reproducible TCR-phosphorylation of GSK3 or FKHR by western blot, but did have success with detection of phospho-S6 by intracellular staining for flow cytometry. Although S6K regulation by PI3K and Akt has been well documented in several systems and is important in T cells, little is known about the use of this mechanism in thymocytes. We find that CD3 cross-linking can induce increases in phospho-S6 levels within 10 minutes of stimulation (Fig. 6D) that are sustained at least 4 hours. $p110_{ABD}$

expression neither increases basal phosphorylation of S6 nor potentiates TCR-induced phosphorylation. We suspect that S6K can only be activated in $CD3^{hi}$ SP thymocytes, since very few cells upregulate S6 phosphorylation. Simultaneous staining for surface antigens worked very poorly but did suggest that this hypothesis is correct.

We are at a loss to explain how $p110_{ABD}$-mediated constitutive Akt activity fails to induce the biochemical changes normally observed downstream of Akt in mature lymphocytes. This suggests either that Akt regulation may be more complex than originally suspected or that Akt function is not an important component of TCR signaling in thymocytes. The first possibility implies that the constitutively active Akt mutants used to date do not accurately represent physiological Akt function. The two lines of mice expressing activated Akt transgenes that have thus far been described do report different phenotypes and biochemical effects. Nor do the biochemical and phenotypic characteristics of $p110_{ABD}$ mice match either one. As discussed above, gagPKB upregulates Bcl-X_L whereas $p110_{ABD}$ does not. On the other hand, myrPKB induces basal Erk activity whereas $p110_{ABD}$ does not (shown below). We thus conclude that although Akt activity indicates that $p110_{ABD}$ functions as a gain-of-function allele for PI3K, phenotypic alterations in $p110_{ABD}$ mice are likely due to Akt-independent mechanisms.

2.3 $p110_{ABD}$ expression potentiates Ca^{++} flux responses

PI3K does activate several Akt-independent pathways downstream of antigen receptors. PH domain-containing Tec kinases (Itk in particular) have been shown to regulate Ca^{++} mobilization via PLCγ and its lipid products (73, 213). The control of PLCγ is complex (reviewed in (214, 215)), but PI3K has been described as a positive regulator (158). Therefore we examined the

effects of $p110_{ABD}$ expression on calcium mobilization after TCR ligation. We utilized the Ca^{++}-binding intracellular dye Indo-I in conjunction with fluorochrome-conjugated antibodies to CD4 and CD8 to monitor changes in intracellular calcium concentration specifically in DP thymocytes. We compared responses of NLC and $p110_{ABD}$ thymocytes to CD3 cross-linking at different concentrations. Figure 6E shows the results of two representative experiments: expression of $p110_{ABD}$ in DP thymocytes strongly potentiates Ca^{++} influx responses to low doses of anti-CD3, without modifying the maximal response. Thus constitutive activation of PI3K improves the ability of DP to flux calcium in response to low intensity stimuli, allowing maximal Ca^{++} flux responses to sub-optimal anti-CD3 antibody concentrations. The maximal response is 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.

We believe that the $p110_{ABD}$-mediated potentiation of Ca^{++} influx is due to a potentiation of Itk activation. This is suggested by the observation that basal levels of intracellular calcium are not elevated in resting $p110_{ABD}$ thymocytes: PI3K activity does not induce Ca^{++} influx in the absence of antigenic stimulation. This correlates with the mode of Itk regulation described so far. All PH domain-containing Tec family kinases require the coordinate signaling of both PI3K and Src kinases to be activated. In T cells, PI3K activity merely induces proper subcellular localization, whereas Lck-mediated phosphorylation activates Itk kinase function. We propose that $p110_{ABD}$-induced PI3K activity allows constitutive association of Itk with the plasma membrane. Thus Itk activation by Lck is potentiated by $p110_{ABD}$ expression. Unfortunately, we were unable to examine Itk phosphorylation in thymocytes. As can be inferred from the rapidity of Ca^{++} influx, Itk is activated extremely rapidly by TCR cross-linking. Maximal Itk phosphorylation is typically observed by 1-2 minutes and quickly returns to basal levels following stimulation (216).

However, we have only been able to establish reliable anti-CD3 stimulations for western blotting with plate-bound antibodies, and this method is not amenable to such short time periods. Despite these limitations, we are confident that this model is correct since a complementary defect in Ca^{++} mobilization is observed in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ cells (182). We suggest that many of the phenotypic alterations induced by $p110_{ABD}$ expression are mediated by enhanced Ca^{++} flux responses to TCR cross-linking.

2.4 $p110_{ABD}$ mice exhibit increased numbers of mature SP thymocytes

The thymii of $p110_{ABD}$ animals have normal cellularity but significantly increased proportions and numbers of the more mature thymic subpopulations, e.g., $CD3^{hi}$ CD4 and CD8 SP thymocytes. Figure 7A shows representative examples of transgenic and NLC thymii from the four lines of mice, demonstrating that each of these populations are nearly doubled in $p110_{ABD}$ animals. All four lines exhibit similar phenotypes, and except where indicated, are used interchangeably throughout this work. We observe a slight, concomitant decrease in the DP compartment, but the percentage of DN cells is unchanged. When we examine the DN compartment more closely, despite a degree of variability from animal to animal, we do not find any significant changes in the distribution among the DN subpopulations DN1 through DN4 induced by $p110_{ABD}$ expression (Fig. 7B). Nor do we observe any $p110_{ABD}$-induced change in the numbers of $\gamma\delta TCR^{+}$ thymocytes. Both $\alpha\beta$T cell lineages are similarly increased in size, as shown by the similar CD4:CD8 ratios observed in the $CD3^{hi}$ compartment of both NLC and $p110_{ABD}$ mice. For the thymii depicted in Figure 7C, these ratios are 2.91 versus 2.51, respectively. Intriguingly, the SP populations are enriched for cells that are more mature and express low levels of HSA (Fig. 7D). HSA expression is not generally disregulated by $p110_{ABD}$, since DP thymocytes from both

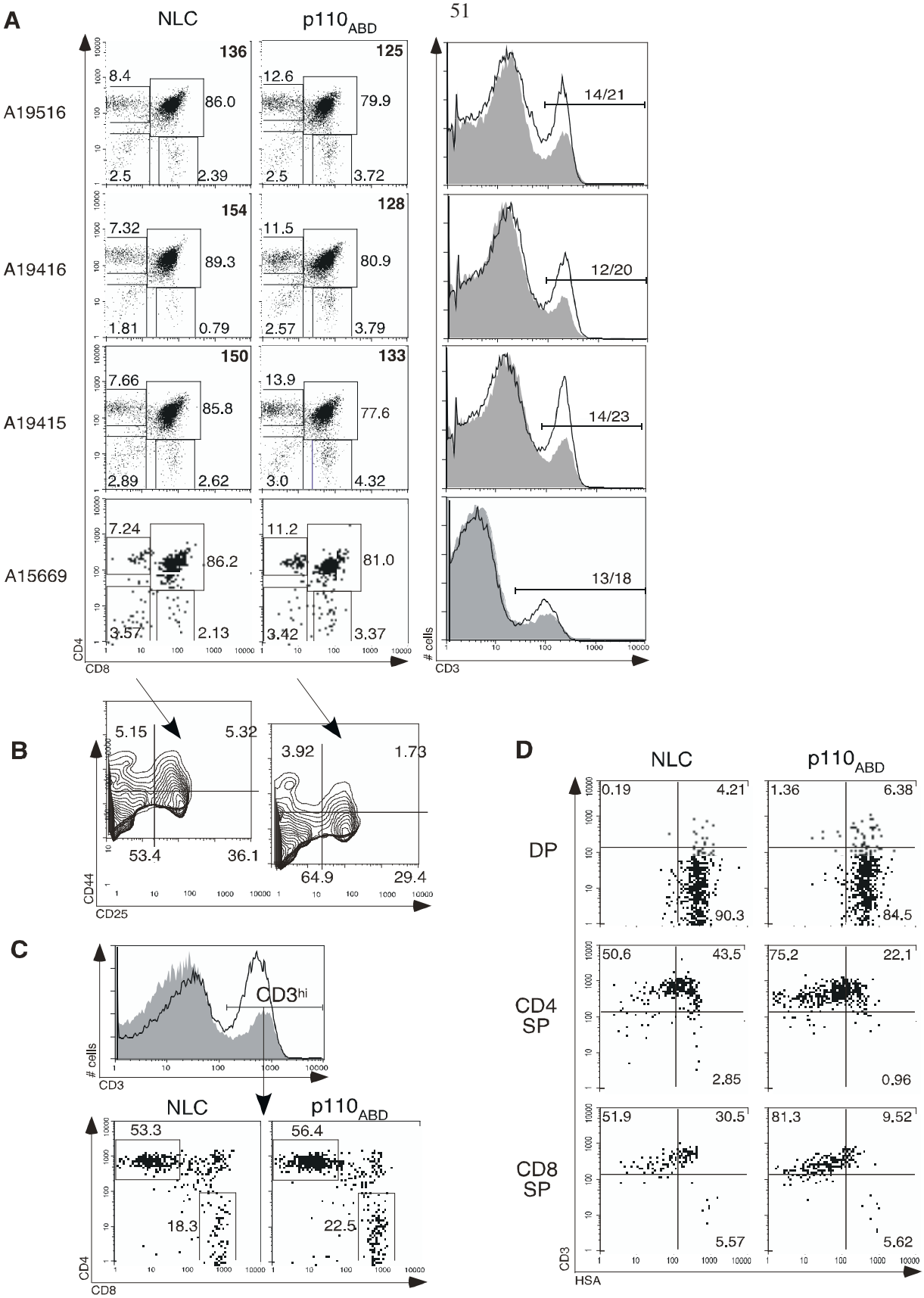

Figure 7. Phenotype of $p110_{ABD}$ mice.

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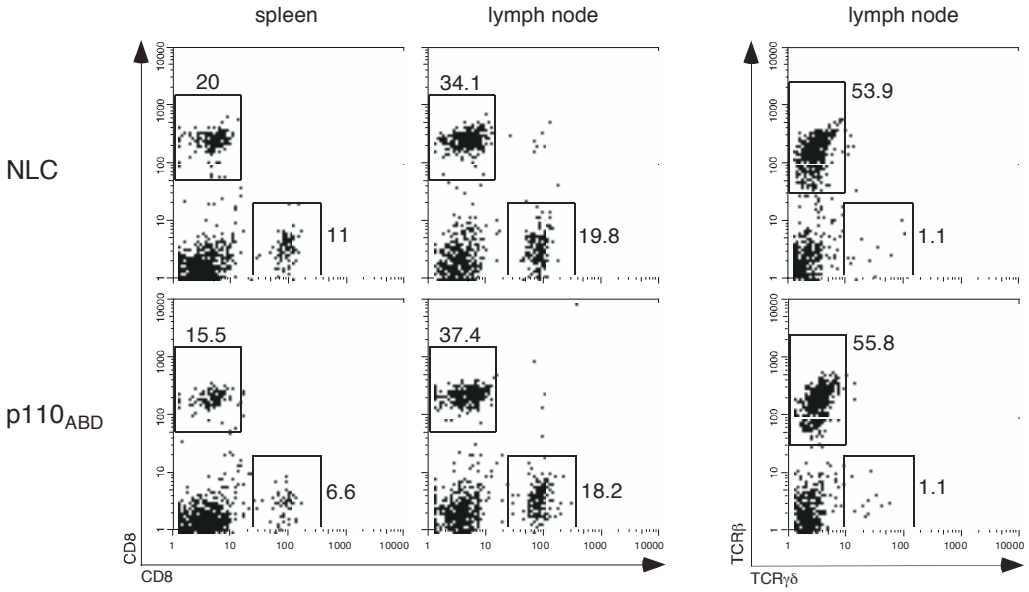

Figure 7. Phenotype of $p110_{ABD}$ mice (continued).

A. The thymii of $p110_{ABD}$ mice contain more SP and $CD3^{hi}$ cells than do NLC. Samples are shown for all four lines generated. For the CD4/8 graphs, numbers next to each region represent the fraction of the thymus, and bold numbers in the top right are the total number of thymocytes. For the CD3 histograms, NLC is represented by grey fill and $p110_{ABD}$ by a black line. The percentage $CD3^{hi}$ is given as NLC/$p110_{ABD}$.

B. DN subset distribution is unaffected by $p110_{ABD}$ expression. Cells from the line 15669 thymii shown in (A) were stained for CD4, CD8, CD25, and CD44. The CD25/CD44 expression patterns of the DN subsets are shown.

C. Both CD4 and CD8 lineage $CD3^{hi}$ cells are increased in $p110_{ABD}$ mice. The CD3 histogram is represented as in (A). The CD4/CD8 plots represent gated $CD3^{hi}$ cells. NLC and $p110_{ABD}$ thymii have similar ratios of $CD3^{hi}$ CD4:CD8 cells.

D. $p110_{ABD}$ mice preferentially accumulate $CD3^{hi}$ HSA^{lo} cells that represent the most mature $\alpha\beta$T subset in the thymus. HSA expression is not generally disregulated, however, since DP cells are still overwhelmingly HSA^{hi}.

E. Peripheral T cells subsets are similar in NLC and $p110_{ABD}$ mice. The slight decreases in $\alpha\beta$T cells shown in $p110_{ABD}$ spleen represent animal-to-animal variation and are not significant. Furthermore, subset representations of CD4 $\alpha\beta$T, CD8 $\alpha\beta$T and $\gamma\delta$Tcells are nearly identical in the mesenteric lymph nodes of both genotypes.

genotypes are overwhelmingly HSA^{hi}. The $CD3^{hi}$ HSA^{hi} population varies from animal to animal: in some mice this population represents a smaller fraction of $p110_{ABD}$ SP cells than of NLC (as shown in Fig. 7D), whereas other $p110_{ABD}$ thymii contain more $CD3^{hi}$ HSA^{hi} cells than NLC. Any increases observed in this population in $p110_{ABD}$ animals are never as large as those for the $CD3^{hi}$ HSA^{lo} compartment, however.

$p110_{ABD}$ transgenic animals display a normal peripheral phenotype. NLC and $p110_{ABD}$ mice have similar numbers of peripheral CD4 and CD8 $\alpha\beta$ T cells and $\gamma\delta$ T cells in both the spleen and mesenteric lymph nodes (Fig. 7E). Furthermore, no transgene-induced differences are observed in the periphery of old mice (6 months 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.

Clearly the levels of activity of PI3K can affect T cell development. The accumulation of mature, $CD3^{hi}$ SP thymocytes that we observe in $p110_{ABD}$ mice could be the result of different processes operating at different stages of development. 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. Alternatively, $p110_{ABD}$ may specifically modulate SP behavior. And a compounded effect involving more than one of these processes is also possible. We therefore undertook experiments to specifically investigate each of these possibilities. Our studies of survival and selection of DP thymocytes will be discussed in the next

chapter, and SP thymocyte characteristics will be examined in Chapter 4.

3. PI3K regulates positive selection independently of negative selection and DP survival

3.1 Possible survival functions of PI3K in DP thymocytes

DP thymocytes have approximately 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 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.

Since DP thymocytes require a strong signal to induce selection and differentiation, they display an enhanced sensitivity to apoptotic stimuli. This is largely due to the downregulation of Bcl-2 expression during the DN to DP transition, at which point thymocyte survival is likely mediated by Bcl-X_L (217). Following successful positive selection, Bcl-2 expression is restored in SP thymocytes (218). Furthermore, forced expression of Bcl-2 in Tg TCR mice restores wild-type numbers of DP thymocytes by sustaining non-selecting cells (219). We therefore sought to determine the expression pattern of Bcl-2 in $p110_{ABD}$ thymocytes. When intracellular Bcl-2 levels are measured by flow cytometry, we find that Bcl-2 expression is restricted to the DN and SP populations of $p110_{ABD}$ animals, as in NLC (Fig. 8A). We also utilized a ribonuclease-protection assay (RPA) kit to assess the expression of multiple Bcl family members. As shown in Figure 8B, NLC and $p110_{ABD}$ thymii express the same amounts of all Bcl factor mRNAs examined: pro-apoptotic genes Bad, Bak, Bax, anti-apoptotic genes Bcl-2, Bcl-W, Bfi1, and the dual-purpose Bcl-X are all expressed equally in NLC and $p110_{ABD}$ thymocytes (Fig. 8B).

Because of the loss of Bcl-2 expression, DP thymocytes may die due to the lack of antigenic

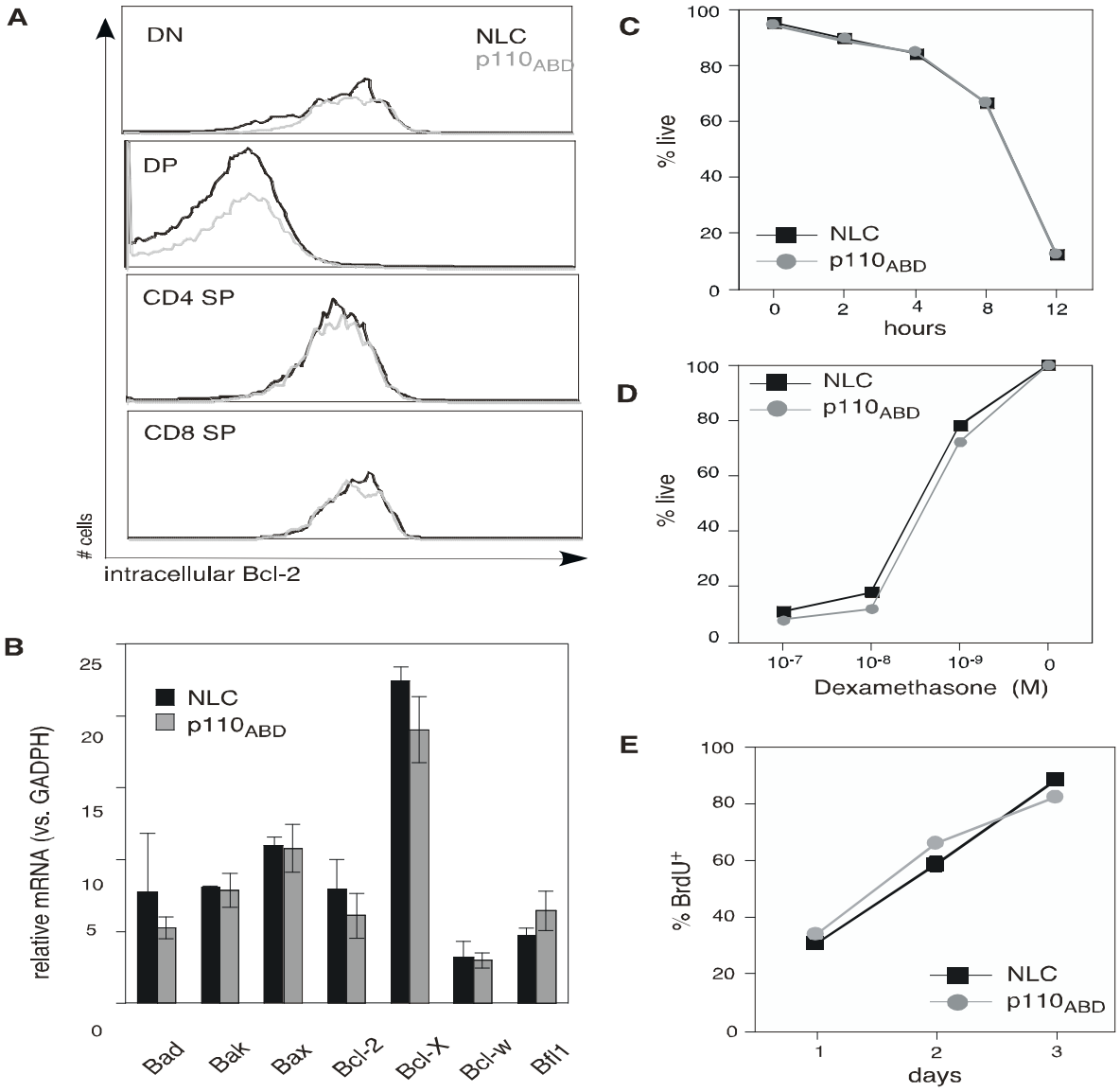

Figure 8. $p110_{ABD}$ expression does not alter DP survival or lifespan.
A. Bcl-2 expression is the same in NLC and $p110_{ABD}$ thymocytes: expression is downregulated in DP thymocytes of both genotypes and upregulated in SP thymocytes.
B. Multiple Bcl-2 family members are expressed at the same level in NLC and $p110_{ABD}$ thymocyes. An RPA kit was used in which multiple probes are used in the same sample, including two housekeeping genes for normalization. The quantity is expressed as a fraction of GADPH in arbitrary units.
C. $p110_{ABD}$ 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.
D. NLC and $p110_{ABD}$ SP thymocytes are equally susceptible to steroid-induced apoptosis. Thymocytes were cultured with varying concentrations of Dexamethasone for 12 hours and then analysed as in (C).
E. 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), 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.

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 improve the viability of thymocytes treated with Dexamethasone, myrPKB has a negligible effect on survival in the absence of stimulation (179, 180). 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. 8C). We also observe identical apoptotic responses when NLC and $p110_{ABD}$ DP thymocytes are cultured for 6 hours with Dexamethasone over a range of concentrations (Fig. 8D).

The survival abilities of thymocytes *in vitro* may not be an accurate representation of that *in vivo*, however. We therefore designed an experiment to examine DP thymocyte life span in which we monitored the transit of thymocytes through the DP compartment using 5-bromodeoxyuridine (BrdU). In young animals the size of the DP compartment is in equilibrium and 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) 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 three days: therefore, after one 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. 8E), demonstrating that $p110_{ABD}$ and NLC DP thymocytes have the same life span.

These results are surprising since $p110_{ABD}$ thymocytes have increased basal Akt activity (Fig. 6A), and there is evidence that DP survival can be regulated by Bad and/or Bcl-X_L via Akt. Jones *et al*. observed upregulation of Bcl-X_L in gagPKB thymocytes that was presumably mediated by

reduced association with Bad (179). Furthermore, overexpression of Bad in thymocytes greatly reduces the total number of thymocytes and the percentages of SP thymocytes (220). But in spite of elevated Akt activity, we did not observe changes in Bad phosphorylation or Bcl-X_L levels in $p110_{ABD}$ thymocytes. Nor do resting $p110_{ABD}$ thymocytes exhibit increased NF-κB DNA-binding activity compared to NLC, ruling out the upregulation of survival genes controlled by this transcription factor. 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 $p65^{PI3K}$ (81), in agreement with our results.

This suggests that although Akt can drive DP thymocyte survival, this may not be a physiological function of PI3K *in vivo.* DP thymocytes appear to survive by a careful balance of the expression of Bad and Bcl-X_L, as apoptotic stimuli induce the upregulation of Bad expression in thymocytes (220). Bad is also upregulated in thymocytes after 5 hours in culture alone, although what drives this seemingly signal-free expression is still unclear (220). Thus it seems that Bad/Bcl-X_L-mediated regulation of DP thymocyte apoptosis occurs at the level of gene expression, rather than via PI3K-induced post-translational events. It should be noted that according to the RPA analysis, in which RNA levels are measured in the same sample, more Bcl-X_L was expressed than Bad. On the other hand, in DN thymocytes PI3K/Akt-mediated Bad phosphorylation is an important event induced by IL-7 treatment (221). This demonstrates that PI3K and its effectors are utilized differently in T cells in a developmental stage-specific fashion.

3.2 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 (36). This system has been used in the past to confirm the role of Erk in positive selection (49). The AND TCR recognizes a fragment of pigeon cytochrome C presented by I-E^k molecules. Thymocytes expressing this MHC class II-restricted TCR are positively selected by I-A^b and I-E^k molecules but not I-A^d (222). Since the $p110_{ABD}$ mice were generated on a C57Bl/6 background, they are H-2^b (I-A^b I-E^-). 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 APC 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) (49).

Expression of the $p110_{ABD}$ transgene significantly improves the positive selection of AND TCR-bearing CD4 thymocytes in H-$2^{b/d}$ animals (Fig. 9 A). 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 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-Tg TCR 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 thymii 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 (49). Our results indicate that expression of $p110_{ABD}$ dramatically improves AND CD4 SP selection in the H-$2^{b/d}$

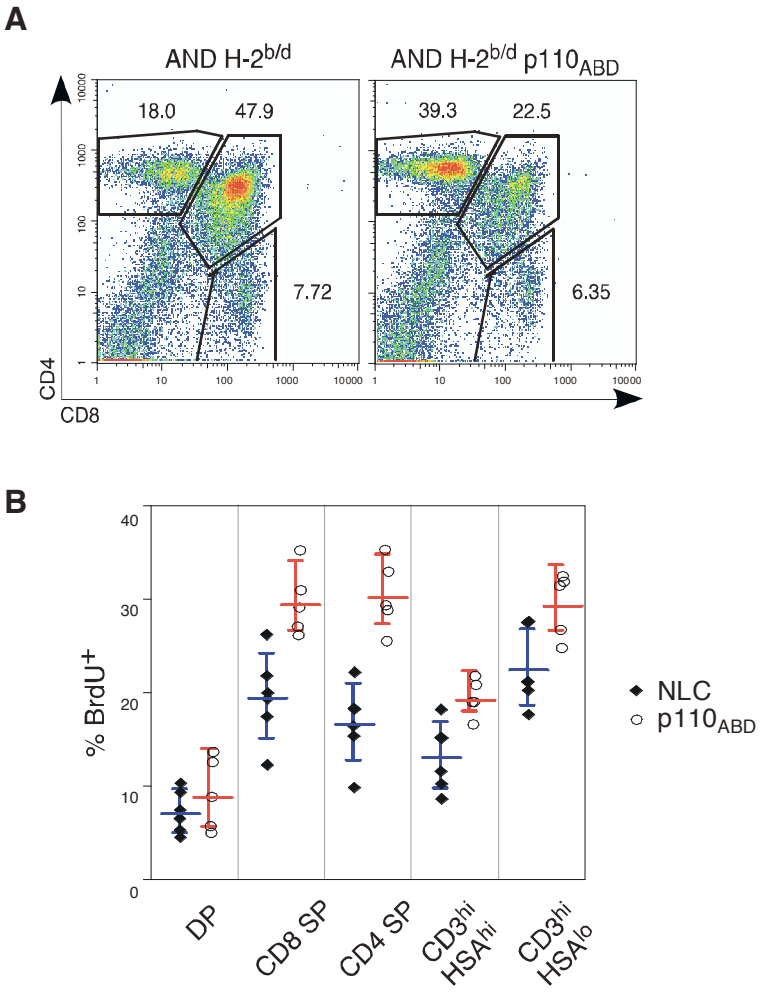

Figure 9. $p110_{ABD}$ expression improves positive selection of AND Tg TCR thymocytes.
A. The AND Tg TCR is positively selected by I-A^b molecules but can not direct thymocyte development on I-A^d: CD4 SP development is suboptimal in the H-$2^{b/d}$ background. However, expression of $p110_{ABD}$ increased the number of CD4 SP thymocytes in AND mice, indicating improved positive selection.
B. 5 days following a pulse labeling of DP thymocytes, more AND H-$2^{b/d}$ $p110_{ABD}$ 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 IP with 1mg BrdU, 3 hours apart. At five days the animals were sacrificed and cells stained for analysis by flow cytometry.

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 be due to mechanisms other than improved positive selection. We therefore performed BrdU pulse-chase experiments to specifically examine the generation of SP thymocytes in the AND H-$2^{b/d}$ background. Cohorts of proliferating cells are labeled with BrdU by intra-peritoneal injection, and the thymii are analyzed by flow cytometry several days later. The graph in Figure 9 B displays the fraction of thymocyte subpopulations that are $BrdU^+$ 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 will have left 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 to AND littermates. This demonstrates that a greater fraction of the compartment is newly generated each day, indicating improved positive selection. In contrast, if the increased CD4 SP populations represent thymocyte accumulation due to an exit defect, the labeled cohort should account for a smaller fraction of the CD4 SP compartment in $p110_{ABD}$ animals compared to non-transgenic littermates. Nor can increased SP proliferation account for the higher fraction of $BrdU^+$ cells, as $p110_{ABD}$ expression does not increase SP division (discussed below in Section 4.1). $BrdU^+$ 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 to non-transgenic animals after 5 days. BrdU tracking thus demonstrates that improved positive selection results in greater BrdU labeling of all mature thymocyte subsets.

3.3 The effect of $p110_{ABD}$ on positive selection is lineage-independent

Recently Rodriguez-Borlado *et al*. published analyses of $p65^{PI3K}$ and $p110\gamma^{-/-}$ mice that show affects on CD4:CD8 differentiation ratios (81). Namely, they observe CD8 fate potentiation in $p110\gamma^{-/-}$ neonates and a reciprocal doubling of CD4 SP in $p65^{PI3K}$ mice. p110γ deficiency even allows more development of CD8 SP thymocytes in the class II-restricted Tg TCR 5CC7 line. On the other hand, they find that $p65^{PI3K}$ expression specifically impairs the development of class I-restricted Tg TCR F5 CD8 SP thymocytes. CD4 SP numbers are not increased in F5 $p65^{PI3K}$ mice, but there are more HSA^- CD4 SP compared to F5. A role for Akt in CD4 lineage-specific positive selection is also suggested by study of the myrPKB transgenic mice. Na *et al*. find that although expression of myrPKB does not alter SP numbers in non-Tg TCR animals, it doubles the fraction of CD4 SP thymocytes in mice expressing the class II-restricted OT-2 Tg TCR (179). MyrPKB can also reverse lineage choice and drive the development of CD4 SP thymocytes in two different class I-restricted Tg TCR lines, OT-1 and HY.

By contrast, 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 Figure 7C, the CD4:CD8 ratio of $CD3^{hi}$ thymocytes in NLC and $p110_{ABD}$ mice is not significantly different: 2.91 vs. 2.51 (respectively) in the animals shown. We have crossed the $p110_{ABD}$ transgene onto three different Tg TCR in total, two class I-restricted and one class II-restricted, and we have observed no change in lineage choices.

There are significant numbers of CD8 SP thymocytes in all AND $H\text{-}2^{b/d}$ mice. Some of these are presumably the "intermediate" CD8 SP (ISP), immature cells progressing from the DN to DP compartments that upregulate CD8 prior to CD4. But most are $CD3^{hi}$, indicating that they are

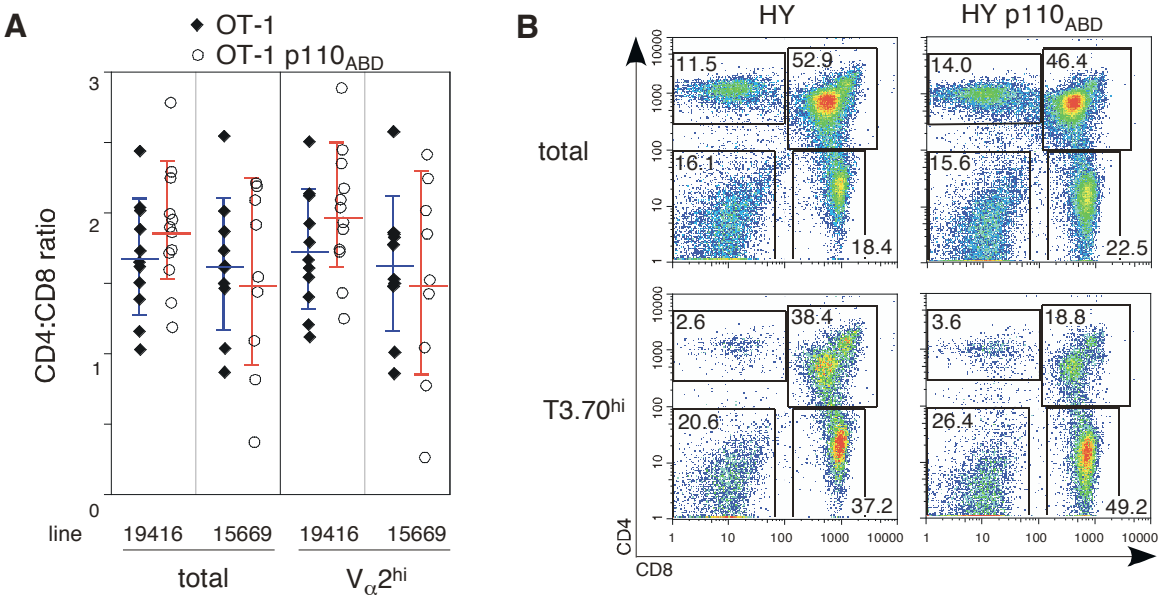

Figure 10. CD4 versus CD8 lineage commitment is unaltered in class I-restricted Tg TCR $p110_{ABD}$ mice

A. $p110_{ABD}$ expression did not significantly alter the CD4:CD8 ratio of total or $V_\alpha 2^{hi}$ thymocytes in OT-1 Tg TCR mice. Slight but insignificant differences were observed between two lines of $p110_{ABD}$ crossed to OT-1. For line 19416, n=11 for both genotypes; for line 15669, OT-1 n=10 and OT-1 $p110_{ABD}$ n=9.

B. HY $p110_{ABD}$ female mice had very small increases in SP fractions compared to HY alone, but the CD4:CD8 ratio was the same for both total thymocytes and Tg TCR $T3.70^{hi}$ thymocytes. Representative animals are shown for each; HY n=3, HY $p110_{ABD}$ n=4.

mature cells that have undergone positive selection. Since these animals were $Rag^{+/-}$, these cells may have rearranged endogenous TCRα chains and been selected by these TCR. Or they may simply be "mismatched," in that coreceptor expression is not correctly matched to the MHC specificity of the TCR, since AND $Rag2^{-/-}$ precursors have been shown to generate TCR^{hi} CD8 SP progeny (223). 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. 9B). The increase in $BrdU^{+}$ SP thymocytes is 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-1 $p110_{ABD}$ animals or in female $p110_{ABD}$ mice expressing the class I-restricted Tg TCR HY. We crossed the OT-1 Tg TCR onto two lines of $p110_{ABD}$ mice, 19415 and 15669. As shown in Figure 10A, there are no significant differences in the CD4:CD8 ratio induced by $p110_{ABD}$ expression. The 19415 line demonstrates very slight increases in the ratio in $p110_{ABD}$-expressing thymii, but these are statistically insignificant. It should also be noted that the populations expressing high levels of the Tg TCR V_{α} chain, $V_{\alpha}2$, exhibit the same CD4:CD8 ratios as for total SP thymocytes. This is not the case in the HY females, however (Fig. 10B). The total SP CD4:CD8 ratios observed for HY and HY $p110_{ABD}$ animals are 0.57 and 0.51 respectively (n=3 and 4). But when only Tg TCR $T3.70^{hi}$ thymocytes are analyzed, these both drop to 0.066 and 0.047. Clearly $p110_{ABD}$ expression does not specifically potentiate CD4 SP development in the MHC class I-restricted Tg TCR systems studied.

3.4 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}) (49). This mutant contains a single amino-acid substitution that mimics a mutation originally identified in *D. melanogaster* genetic screens. The substitution results in delayed dephosphorylation of Erk, and therefore the mutant is hypersensitive to activation rather than constitutively active. Intriguingly, Erk was one of the first kinases shown to be inhibited in anti-CD3 stimulated T cells treated with Wortmannin (224, 225). PI3K-mediated Erk activation is also suggested by the impaired TCR-induced Erk phosphorylation that is observed in p110δ^{D910A} mutant mice (178). Furthermore, Erk is constitutively phosphorylated in resting PTEN-deficient peripheral T cells (82). How this regulation could be mediated, whether via Akt or Itk, remains unclear, however. Resting thymocytes from myrPKB animals have higher levels of phospho-Erk than wild-type cells, and CD3-induced Erk activation lasts significantly longer in transgenic thymocytes (180). On the other hand, Tec kinases may regulate Erk activity since these kinases can activate PLCγ, thereby catalyzing the production of DAG, which in turn activates the Ras-specific GTP exchange factor RasGRP. Indeed, CD3-induced Erk phosphorylation is significantly impaired in Itk$^{-/-}$ and Rlk$^{-/-}$ Itk$^{-/-}$ thymocytes (86). 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. 11A), and both display the same sensitivity to PMA treatment. Unfortunately, the antibodies used for flow cytometric detection of intracellular phospho-Erk cross-react with the 2C11 anti-CD3 antibody used for thymocyte stimulations. We therefore assessed CD69 expression as a representation of the Erk activity

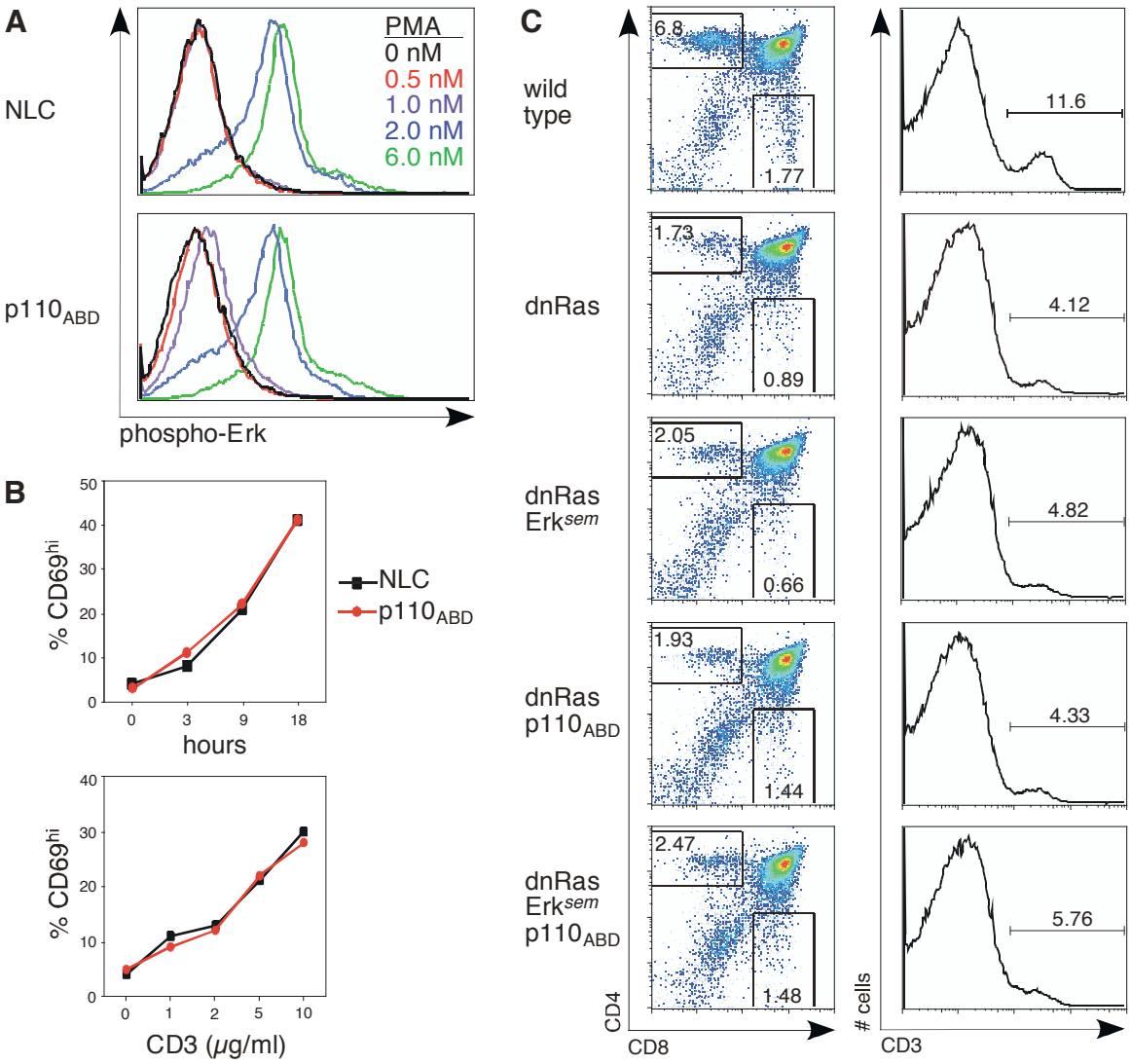

Figure 11. $p110_{ABD}$ expression does not upregulate Erk activity or restore dnRas thymocyte development.

A. 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 upregulated 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) and CD69 expression assessed by flow cytometry at 3, 9, and 18 hours. The bottom graph represents CD69 expression induced by anti-CD3 treatment for 9 hours over a range of concentrations.

C. SP and $CD3^{hi}$ thymocyte development is severely impaired by expression of a dominant-negative Ras (dnRas). Neither a hypersensitive Erk (Erk^{sem}) nor $p110_{ABD}$ expression alone can restore SP development. Expression of both Erk^{sem} and $p110_{ABD}$ does not rescue positive selection either, indicating that at least a third Ras effector pathway is required.

induced by CD3 cross-linking, since Ras activity can direct upregulation of this marker (226). We performed a checkerboard analysis in which thymocytes were stimulated for 3, 9, and 18 hours over a range of concentrations. Two analyses of these experiments are shown in Figure 10B. There are no detectable $p110_{ABD}$-induced differences in the kinetics of CD69 upregulation or in the dose response curve. Thus although $p110_{ABD}$ can potentiate calcium flux responses, and therefore concomitant DAG production, this does not activate the RasGRP/Ras/Erk pathway in DP thymocytes. We therefore conclude that PI3K can promote positive selection via an Erk-independent pathway.

The Erk-independence of $p110_{ABD}$-induced positive selection suggests that positive selection may result from the integration of Erk and PI3K signals. This is intriguing to note since defects in positive selection induced by the expression of a dominant-negative Ras cannot be rescued by Erk^{sem} expression (JAI, unpublished results, and Fig. 11C). Multiple studies have suggested that PI3K can function as a Ras effector, but this role has not been conclusively proven in lymphocytes. Ten years ago expression of the oncogenic vRas was shown to activate PI3K in *S. pombe* and COS cells (227, 228). Expression of a dominant negative Ras in PC12 cells is sufficient to block PI3K activity as well, whereas expression of dominant negative PI3K (Δp85) is sufficient to block H-RasV12-induced transformation of NIH 3T3 cells (228, 229). The catalytic subunits of PI3K do interact with Ras in a GTP-dependent fashion (230). This interaction appears to induce an activating conformational change, since a point mutation of the critical binding residue can "mimic" Ras function and induces basal PI3K activity in COS cells (230). Also, p85-mediated inhibition of p110 catalytic activity may be due to prevention of p110 association with Ras (231). However evidence suggests that PI3K does not function as a Ras effector in T cells. H-RasV12-induced AP-1 activity in Jurkat cells is insensitive to Ly294002 treatment at concentrations that effectively inhibit Akt activity (232). H-RasV12 mutants have

also been developed with mutations that restrict effector activation: PI3K is the only effector activated by H-RasV12C40, and expression of this mutant fails to induce Ras-mediated primary CD4 T cell activation events (145). PI3K may be activated downstream of Ras in T cells by chemokine receptors, however. Transfection of H-RasV12C40 into resting peripheral T cells can induce LFA-1 affinity changes and ICAM-1 binding normally associated with chemotactic stimulation (233).

In light of these reports, we attempted to determine if PI3K mediated Ras effects in selection. To do so we used mice expressing the dominant negative mutant H-RasN17, which bears a single amino-acid substitution within the effector loop, referred to as dnRas. We crossed the $p110_{ABD}$ transgene onto this line, but this fails to restore normal numbers of SP and $CD3^{hi}$ thymocytes (Fig. 11C). We also generated triply-transgenic mice that express dnRas, Erk^{sem}, and $p110_{ABD}$, but even these animals have decreased mature thymocyte populations. Based on these results we cannot be sure if PI3K functions as a Ras effector in thymocytes. Regardless, from this we can conclude that the Ras effector signals needed for efficient positive selection require an additional pathway distinct from Erk and PI3K.

3.5 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 three model systems. For one, we observed deletion of $p110_{ABD}$ thymocytes *in vivo* by viral superantigens (vSAG). All mouse strains are endogenously infected with a variety of mouse mammary tumor viruses (MMTVs) that express viral superantigens (vSAG) ubiquitously. vSAG constitutively cross-link

I-E molecules with specific TCR V_β segments irrespective of the peptide bound by MHC (Fig. 12). This constitutes a strong TCR signal, inducing the deletion of thymocytes bearing such segments (234). C57Bl/6 mice are endogenously infected with MMTV8 but do not express I-E, and thus retain thymocytes utilizing the V_β segments recognized by vSAG8, V_β11 and 12. In addition to MMTV8, DBA/2J mice carry MMTV7, and vSAG7 recognizes V_β6, 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 x C57Bl/6 F_1 mice these populations are deleted by vSAG7 and 8 (Fig. 12A). The $p110_{ABD}$ transgene does not rescue the development of these populations in DBA/2J x C57Bl/6 F_1 mice.

Superantigen-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-1. The OT-1 TCR recognizes an ovalbumin peptide (SIINFEKL) in the context of the MHC class I molecule $H\text{-}2K^b$ (29). The co-culture of OT-1 thymocytes with antigen presenting cells (APC) presenting SIINFEKL provides an *in vitro* model for negative selection that is dose-dependent (45). OT-1 and OT-1 $p110_{ABD}$ thymocytes were cultured with APC loaded with different concentrations of SIINFEKL and analyzed after 18 hours. DP thymocytes from both OT-1 and OT-1 $p110_{ABD}$ are efficiently deleted in a dose-dependent fashion, with an LD_{50} of 10^{-10}M peptide. In individual experiments there appear to be slight increases in OT-1/$p110_{ABD}$ DP thymocyte survival relative to OT-1 thymocytes at high peptide concentrations. However, statistical analyses of the data from multiple deletion assays (n=14), normalized so that the DP survival rate is expressed as a percentage of the survival in the absence of peptide, shows that the difference is not significant

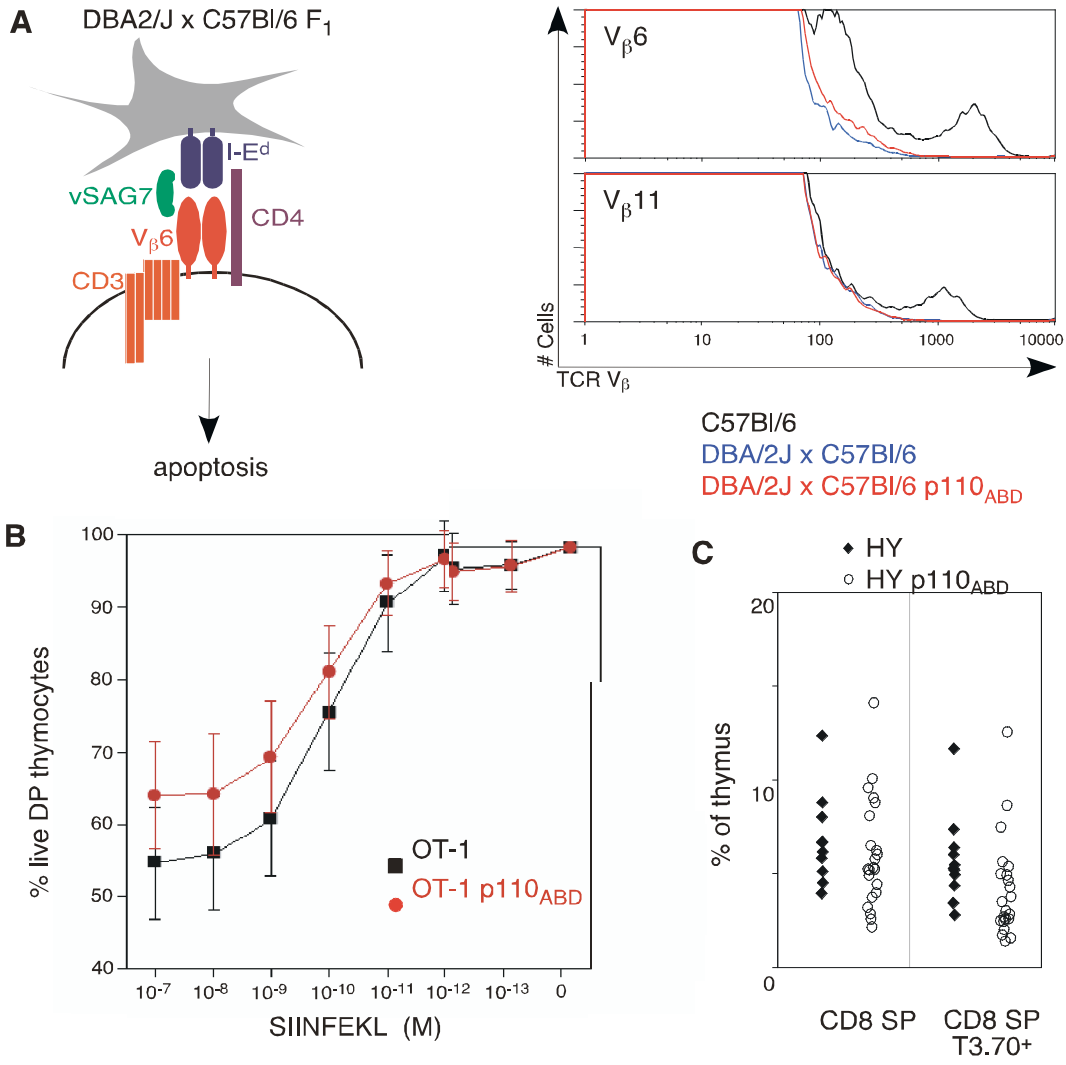

Figure 12. Negative selection is unaffected by $p110_{ABD}$ expression *in vitro* and *in vivo*.
A.The $p110_{ABD}$ transgene does not protect thymocytes from stringent, superantigen-mediated negative selection. $p110_{ABD}$ mice (endogenously infected with MMTV8 but lacking I-E^b) were crossed with DBA/2J mice (MMTV7$^+$ I-E^+). In the F_1 progeny, SAGs 7 and 8 crosslink I-E molecules to TCR V_β6 and V_β11, 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 F_1 mice.
B. OT-1 and OT-1 $p110_{ABD}$ DP thymocytes surive the same in an *in vitro* deletion assay. The $p110_{ABD}$ transgene was crossed onto the OT-1 transgenic line expressing a TCR specific for the SIINFEKL peptide, and thymocytes were co-cultured for 18 hours 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-1 $p110_{ABD}$ DP may survive slightly better than OT-1 at high concentrations of peptide, the improvement is not significant when the data from multiple experiments are combined. The graph includes data from 8 independent experiments, each performed in duplicate (n=14 for each genotype).
C. The HY Tg TCR recognizes a male-specific peptide presented by MHC class I molecules, and CD8 SP cells are negatively selected in male HY mice. CD8 SP and Tg TCR T3.70^{hi} CD8 SP cells constitute the same fraction of the thymus in HY and HY $p110_{ABD}$ males.

(Fig. 12B). This result confirms the failure of $p110_{ABD}$ to alter negative selection.

We also attempted to assess the effect of $p110_{ABD}$ expression on the deletion of HY Tg TCR thymocytes in male animals. Since this Tg TCR recognizes a male-specific antigen, CD8 SP cells are efficiently deleted in male mice. Unfortunately, we observe sizable numbers of CD8 SP and Tg TCR $T3.70^{hi}$ CD8 SP thymocytes in HY males that do not express the $p110_{ABD}$ transgene (Fig. 12C). We are not certain exactly why this is but suspect it may be related to a non-specific background effect: these mice were not backcrossed to C57Bl/6 for a full 12 generations. HY $p110_{ABD}$ males appear to have similar numbers of these thymocytes as well (Fig. 12C), but we are reluctant to make any assertions about the effect of $p110_{ABD}$ expression since negative selection is so inefficient in the control HY animals.

3.6 A model for PI3K function in thymocyte selection

We conclude that PI3K activity can specifically enhance positive selection in a lineage-independent fashion without affecting basal DP survival or negative selection. These results agree most closely with the descriptions of Tec kinases in DP thymocytes. Mice deficient for Itk or for Itk and Rlk exhibit impaired positive selection (85-87). However, the role of Itk in lineage commitment remains unclear. Itk may be more important for CD4 lineage development, since Itk-null animals have normal numbers of peripheral CD8 T cells but reduced CD4 T cell numbers (87). However, CD8 SP development in class II-restricted Tg TCR backgrounds is not potentiated by Itk deficiency as it is by loss of p110γ (81, 87). Development of HY Tg TCR CD8 SP is inhibited in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ mice as well, arguing that Itk is required for positive selection of both lineages. Since we observe $p110_{ABD}$-potentiated Ca^{++} flux responses to TCR

cross-linking of DP thymocytes, we propose that PI3K-mediated activation of Itk and downstream Ca^{++} responses are critical components of positive selection signals.

Whereas $p110_{ABD}$ expression does not modify negative selection, on the other hand Itk appears to be an important component of this process. The requirement for Itk for efficient negative selection was first observed in HY Tg TCR male animals, in which the numbers of CD8 SP thymocytes and peripheral CD8 T cells are increased in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ mice (85, 86). The same effect was observed in the CD4 lineage in a set of elegant experiments by Leslie Berg's lab (Lucas, *et al*. (200)). They crossed $Itk^{-/-}$ animals expressing Tg TCR that use $V_\beta3$ (2B4, 5CC7, and AND) to the 129 line, in which all $V_\beta3^+$ thymocytes are deleted by endogenous SAG. Tg TCR thymocyte numbers are greatly reduced by SAG expression, but this reduction is less in $Itk^{-/-}$ mice (200). Specifically, SAG expression induces a 880-fold reduction in the number of DP thymocytes in 2B4 $Itk^{+/-}$ mice but only a 90-fold reduction in 2B4 $Itk^{-/-}$ animals. Lucas *et al*. also examined peptide antigen-specific deletion in 5CC7 Tg TCR mice. Inducible promoter-driven expression of a fusion protein of hen egg lysozyme and cytochrome C (HEL-cyt) drives deletion of 5CC7 CD4 SP thymocytes in a dose-dependent fashion (200). Although Itk deficiency reduces CD4 SP numbers due to impaired positive selection, HEL-cyt expression induces a smaller decrease in the CD4 SP compartment than in Itk-competent thymii. These experiments do suggest a role for Itk in negative selection *in vivo*.

One important feature of the selection defects in $Itk^{-/-}$ mice is the relationship between the severity of the effect with the avidity of the TCR/ligand interaction. This is especially evident in the impaired positive selection of class II-restricted Tg TCR. When AND CD4 SP development is assessed in $Itk^{-/-}$ mice on different MHC backgrounds, the defect in positive selection is most severe in the more weakly-selecting $H\text{-}2^{b/b}$ background (87). Only minor decreases are observed

on the strongly selecting H-$2^{k/k}$ background, whereas Itk deficiency induces an intermediate decrease in CD4 SP thymocytes and peripheral CD4 T cells in the mixed H-$2^{k/b}$ background. Of the Tg TCR examined in the H-$2^{k/k}$ background, the decrease in CD4 SP numbers induced by Itk deficiency correlates with the avidities of TCR interactions with I-E^k: AND>5CC7>2B4 (87). Intriguingly, the same hierarchy is observed in SAG-mediated deletion of Tg TCR thymocytes. SAG expression induces 880- and 9-fold decreases of the numbers of DP thymocytes in 2B4 and 5CC7 mice that are Itk-competent, respectively (200). In $Itk^{-/-}$ mice, the decreases are only 90- and 1.5-fold. These results strongly indicate the existence of signaling thresholds that regulate selection processes.

These effects appear to relate to the slower and reduced intensity TCR signaling in $Itk^{-/-}$ thymocytes (87, 200). AND $Itk^{-/-}$ mice accumulate $CD69^{hi}$ DP and SP thymocytes, suggesting that Itk-null cells do not progress through positive selection as efficiently as cells with fully competent Itk function (87). Furthermore, Lucas *et al.* describe higher numbers of DP thymocytes in 5CC7 and AND Tg TCR SAG^+ mice that are $Itk^{-/-}$ versus $Itk^{+/-}$, which they attribute to delayed negative selection (200). When CD5 expression is measured as a surrogate for TCR signal intensity, Itk-null DP and Tg TCR^{hi} CD4 SP thymocytes are found to express lower amounts of CD5 (87). Attenuated TCR signals are likely due to impaired activation of PLCγ in $Itk^{-/-}$ cells (86, 235). $Itk^{-/-}$ thymocytes flux Ca^{++} in response to TCR cross-linking as quickly as wild-type cells to the same degree, but the sustained intracellular Ca^{++} concentration is substantially lower (86). Presumably it is this sustained increase in intracellular Ca^{++} levels that mediates selection processes.

If Itk-induced Ca^{++} flux responses mediate negative selection, it must occur via a pathway other than the activation of calcineurin. Modification of calcineurin activity in thymocytes, either by

expression of a constitutively active mutant or by knock-out of the regulatory subunit, causes alterations of positive selection only, without changes in multiple models of negative selection (73, 236). Ca^{++}-induced pathways have been implicated in negative selection before, however. Expression of a dominant-negative Nur77 blocks negative selection in two Tg TCR models, F5 and male HY mice, but fails to alter positive selection (72, 237). The promoter of Nur77 contains two Ca^{++}-responsive elements to which MEF2 can bind (74). How Ca^{++} regulates Nur77 transcription remains unclear, as it may or may not involve calcineurin. Calcineurin dependency is suggested by sensitivity of Nur77 expression to treatment with the calcineurin inhibitor Cyclosporin A (74). On the other hand, Nur77 expression appears to be regulated by Ca^{++}-mediated antagonism of the factor Cabin1 that is insensitive another calcineurin inhibitor, FK506 (238).

Integration of these results suggests that upon TCR cross-linking in DP thymocytes, PI3K-mediated Itk activity directs PLCγ activation and Ca^{++} flux. The Ca^{++} response is a bifurcation point at which positive and negative selection functions are separable: Ca^{++}-mediated activation of calcineurin contributes to positive selection whereas a calcineurin-independent pathway directs negative selection, possibly via Nur77 and its homologs. There is likely an added level of specificity dictated by signal strength, whereby lower intensity signals drive positive selection by activating only calcineurin. High-avidity TCR-ligand interactions induce a higher, more sustained Ca^{++} flux that allows activation of the negative selection program. This signal threshold model is supported by the observations of Tec kinase deficiencies in HY Tg TCR males, in which selection is switched from negative to positive by loss of Rlk and Itk (86). A threshold model can also be used to explain the restriction of $p110_{ABD}$ effects to positive selection. $p110_{ABD}$ expression induces a modest potentiation of Ca^{++} signals that is sufficient to improve positive selection. But no significant changes to the sustained intracellular Ca^{++} concentration are observed in $p110_{ABD}$

mice, indicating that signal strength is not increased sufficiently to alter negative selection.

4. A role for PI3K in thymic exit

4.1 PI3K activity in SP thymocytes

We observe that SP thymocytes of $p110_{ABD}$ animals exhibit a significantly higher fraction of the most mature SP $CD3^{hi}$ HSA^{lo} thymocyte population compared to NLC: 75.2% and 81.3% of CD4 SP and CD8 SP, respectively, in $p110_{ABD}$ versus 50.6% and 51.9% in NLC (Fig. 7D). HSA expression is not generally disregulated in $p110_{ABD}$ thymocytes, as the DP cells of both NLC and $p110_{ABD}$ are overwhelmingly HSA^{hi} (Fig. 7D). These observations convinced us to closely examine on the effect of $p110_{ABD}$ in SP thymocytes.

Newly-selected SP thymocytes are quiescent, but shortly thereafter they can proliferate. CFSE-labeled $CD69^{+}$ SP and their progeny can undergo up to 6 cycles of division in reaggregate FTOC experiments, but most cells divide only 1-3 times (239). This proliferation is driven by MHC class II^{+} thymic stroma, but does not require interaction with the selecting ligand since $H\text{-}2^{d}$ stroma supports maturation and proliferation of $H\text{-}2^{b}$ thymocytes (239). The timing of the proliferation remains unclear, however, since BrdU labeling studies suggest instead that division occurs immediately prior to thymic exit (240). $BrdU^{+}$ SP cells analyzed two hours after pulse labeling are highly enriched for the most mature phenotype, HSA^{lo} $Qa\text{-}2^{hi}$ (240). When FITC is injected intrathymically 4 hours after BrdU labeling and then peripheral organs analyzed 16 hours later, recent thymic emigrants ($FITC^{+}$) contain more $BrdU^{+}$ cells than resident populations ($FITC^{-}$) (240). From this result it is concluded that at least 10% of emigrants are just-post-cycling cells and that 30% of the cycling SP in the thymus are ready to emigrate in the next few hours. When the time between BrdU and FITC injections was increased to 24 hours, Le Campion *et al.* estimated that at least 50% of mature cells exiting the thymus have divided in the last 24 hours

(241). According to the BrdU studies, cycling SP have downregulated surface CD69 but express varying levels of CD62L (241).

Although Akt has primarily elicited interest due to its survival-inducing effects, there is also significant evidence to suggest that PI3K and Akt can regulate cell cycle progression and proliferation. One of the initial characterizations of Akt was that of the oncogenic vAkt, and the embryonic lethality of $p110\alpha^{-/-}$ mice is due to defects in proliferation rather than increased apoptosis (193, 242). Lymphoproliferative disorders and lymphomas are described in $PTEN^{+/-}$ and $p65^{PI3K}$ mice (243-245). Strikingly, myrPKB expression in an IL-2-dependent T cell lymphoma line is sufficient to prevent G_1 arrest following IL-2 withdrawal (246). PI3K couples the pro-mitotic function of E2F to the IL-2 receptor: expression of Δp85 blocks IL-2-induced activity of this transcription factor, whereas a constitutively active p110 construct induces IL-2-independent E2F activity (247). Furthermore, Akt can regulate multiple aspects of cell-cycle progression via inhibition of forkhead transcription factors. Forkhead factors are transcriptional repressors of $p27^{kip1}$ and pro-mitotic cyclins (e.g., cyclin B), and they activate the transcription of anti-mitotic cyclins (e.g., cyclin G2) (248-250). PI3K can also regulate translation of cyclin D via S6K (251). It is thus possible that PI3K and Akt can regulate cell cycle control in thymocytes as well.

Even one additional round of proliferation would be sufficient to yield the population increases we observe in $p110_{ABD}$ mice. We therefore assessed $p110_{ABD}$ SP thymocyte proliferation by measuring DNA content of $CD3^{hi}$ cells. However both $CD3^{hi}$ HSA^{hi} and $CD3^{hi}$ HSA^{lo} populations contain the same proportion of cells in the S/G_1 phase in NLC and $p110_{ABD}$ animals (Fig 13A). Intriguingly, we find that more of the earlier HSA^{hi} cells are dividing compared to the late HSA^{lo} cells. This correlates with the proposed post-selection division rather than the model of late, pre-

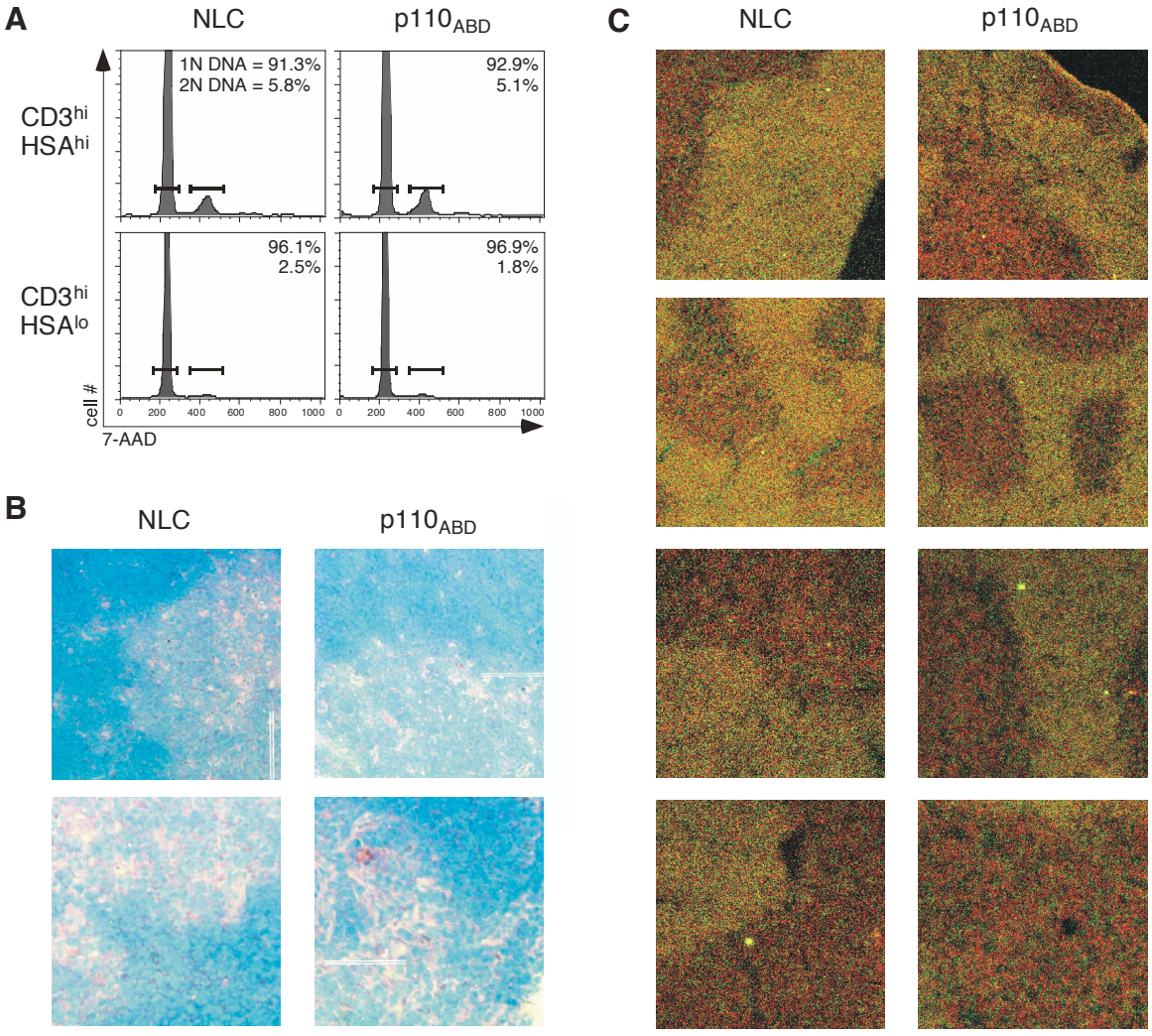

Figure 13. $p110_{ABD}$ expression does not increase SP proliferation or alter SP thymic localization.

A. The same fraction of NLC and $p110_{ABD}$ mature thymocytes are in cell cycle. DNA content was analysed by 7AAD staining in combination with surface antigen antibodies

B. $p110_{ABD}$ thymii have a grossly normal thymic architecture. Discrete medullar domains are observed in $p110_{ABD}$ thymii (the light blue areas), and the CMJ of NLC and $p110_{ABD}$ are similarly constructed. Cryosections were stained with Giemsa and Jenners stains.

C. DP and SP thymocytes localize to cortical and medullary regions, respectively, in NLC and $p110_{ABD}$ thymii. CD4 (red) and CD8 (green) SP thymocytes are found in small clusters evenly distributed throughout the medulla, whereas DP cells (yellow) are confined to the cortex. $p110_{ABD}$ expression does not increase the number of cortical SP, nor do $p110_{ABD}$ SP accumulate at the CMJ. Cryosections were stained with CD8-FITC and CD4-PE anitbodies and then visualized with a laser confocal microscope.

exit cycling.

We were also curious as to whether the alterations in $p110_{ABD}$ subsets correlate with disrupted thymic architecture. When we examine Giemsa stains of thymus sections, we find that the thymii of transgenic and NLC animals are similarly organized into discrete cortical and medullary zones. As shown in Figure 13B, the cortico-medullary junctions (CMJ) of $p110_{ABD}$ animals resemble those of NLC. We also find that the locations of T lineage cells are the same: SP thymocytes complete maturation in the medulla and then return to the CMJ to exit via venules. Dual immunofluorescence staining with CD4 and CD8 antibodies reveals similar distributions of DP (yellow) and SP (CD4 red, CD8 green) in both genotypes (Fig. 13C). The medullas in $p110_{ABD}$ animals appear to be more tightly packed with SP cells than NLC mice, but these cells are relatively evenly distributed. Importantly, clusters of $p110_{ABD}$ SP cells are not observed in the cortex and do not accumulate at the CMJ.

4.2 $p110_{ABD}$ expression delays the appearance of peripheral T lymphocytes

In adult $p110_{ABD}$ 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 Figure 14A, the total thymus size increases at the same rate in both NLC and $p110_{ABD}$ animals. Likewise, the accumulation of $TCR\beta^{hi}$ SP thymocytes does not occur until after the first month of life. However, αβ T cells appear in the spleen (Fig 14B) slower in $p110_{ABD}$ animals compared to NLC. The delay in peripheral colonization is observed in both CD4 and CD8 T cell lineages at similar levels. By contrast, B cell colonization of the periphery was unchanged by $p110_{ABD}$

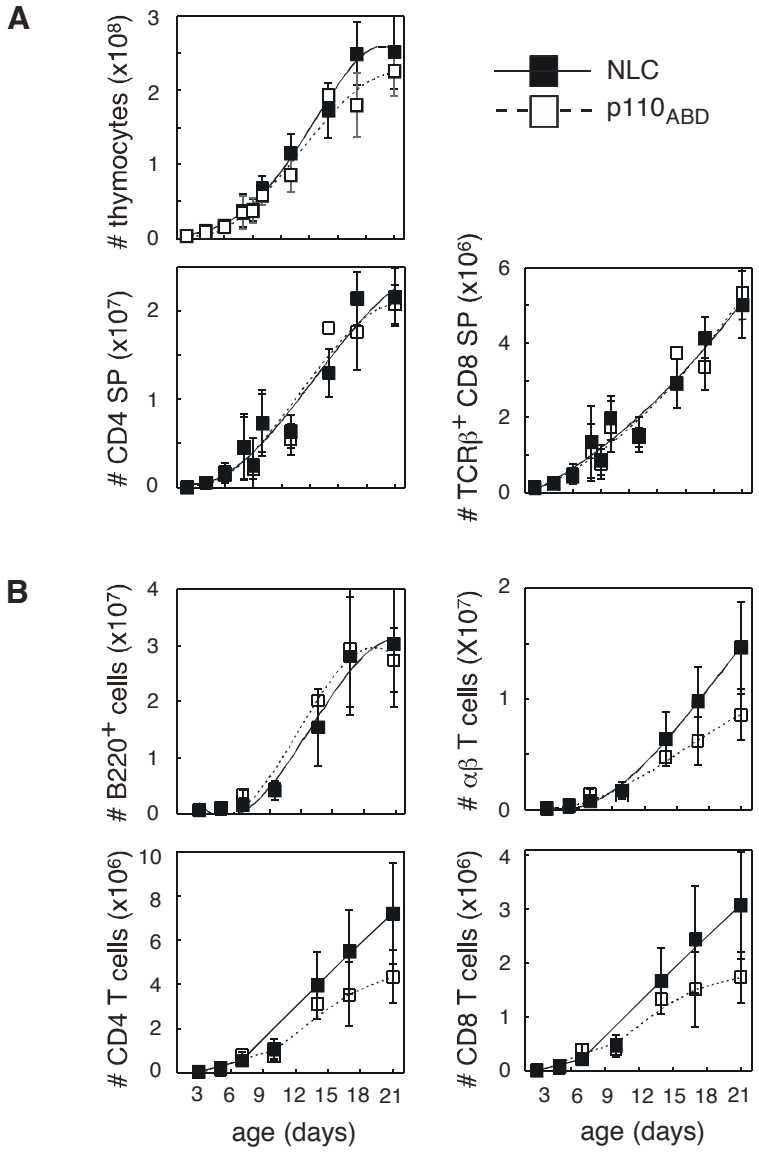

Figure 14. Mature T lymphocytes colonize the periphery of $p110_{ABD}$ neonatal mice slower than NLC.

A. Neonatal thymocytes develop at the same rate in $p110_{ABD}$ (open squares, dotted lines) and NLC (closed squares, solid lines) animals. Animals were sacrificed at the times indicated, and thymocytes stained with CD8-FITC, CD4-PE, CD3-CyChrome, and TCRβ-APC. The number of mature CD8 SP was determined by only counting cells that were $TCR\beta^{hi}$, thus excluding $TCR\beta^{lo}$ ISPs. For each timepoint (n = 1 to 17).

B. Although the spleens of neonatal mice are colonized by $B220^+$ B cells at the same rate in NLC and $p110_{ABD}$ animals, the appearance of both CD4 and CD8 lineages of αβ T cells are delayed in transgenic mice. Samples were stained with TCRγδ-FITC, B220-PE, and TCRβ-APC.

expression. Identical results are observed in mesenteric lymph nodes.

To verify that this difference is also present in adult thymic development rather than a characteristic specific to fetal thymopoiesis, we devised a competitive adoptive transfer scheme to compare the emigration kinetics of wild-type (WT) and $p110_{ABD}$ thymocytes. We prepared bone marrow cells from nontransgenic Ly5.1 Thy1.2 C57Bl/6 animals and Ly5.2 Thy1.2 $p110_{ABD}$ animals. After depleting these preparations of $CD3^+$ T cells, we mixed cells at a 1:1 ratio and injected 10^6 into lethally-irradiated Ly5.2 Thy1.1 recipients. At subsequent timepoints of analysis, the two transferred populations and host cells are discriminated via Ly5.1 and Thy1.1 expression (Fig. 15A, top row): wild-type cells are $Ly5.1^+$ $Thy1.1^-$, and $p110_{ABD}$ cells are $Ly5.1^-$ $Thy1.1^-$, whereas host cells are $Ly5.1^-$ $Thy1.1^+$. 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 $p110_{ABD}$-derived lymphocytes. As shown in Figure 15A, thymic colonization occurrs at the same rate without any reproducible accumulation of $p110_{ABD}$ SP cells compared to wild-type. Likewise, B cells efficiently develop from precursors of both genotypes and colonize the spleen (Fig. 15B) and mesenteric lymph nodes at the same rate. In marked contrast, there is a significant delay in the appearance of $p110_{ABD}$-derived mature $\alpha\beta$ T cells in the periphery. Wild-type T cells are readily observable by 3 weeks post-transfer, whereas $p110_{ABD}$ T cells do not appear until after 6 weeks. Even by 4 months post-transfer, the ratio of $p110_{ABD}$ T cells to B cells has not reached the same value as wild type-derived cells. It should be noted that this imbalance in T:B ratio is observable even in animals in which the $p110_{ABD}$ cells engrafted better than nontransgenic cells (see 6 week timepoint in Fig. 15B).

We believe that the eventual appearance of $p110_{ABD}$-derived T cells is due to delayed but successful thymic exit. We do not think that these cells arise by homeostatic proliferation of a

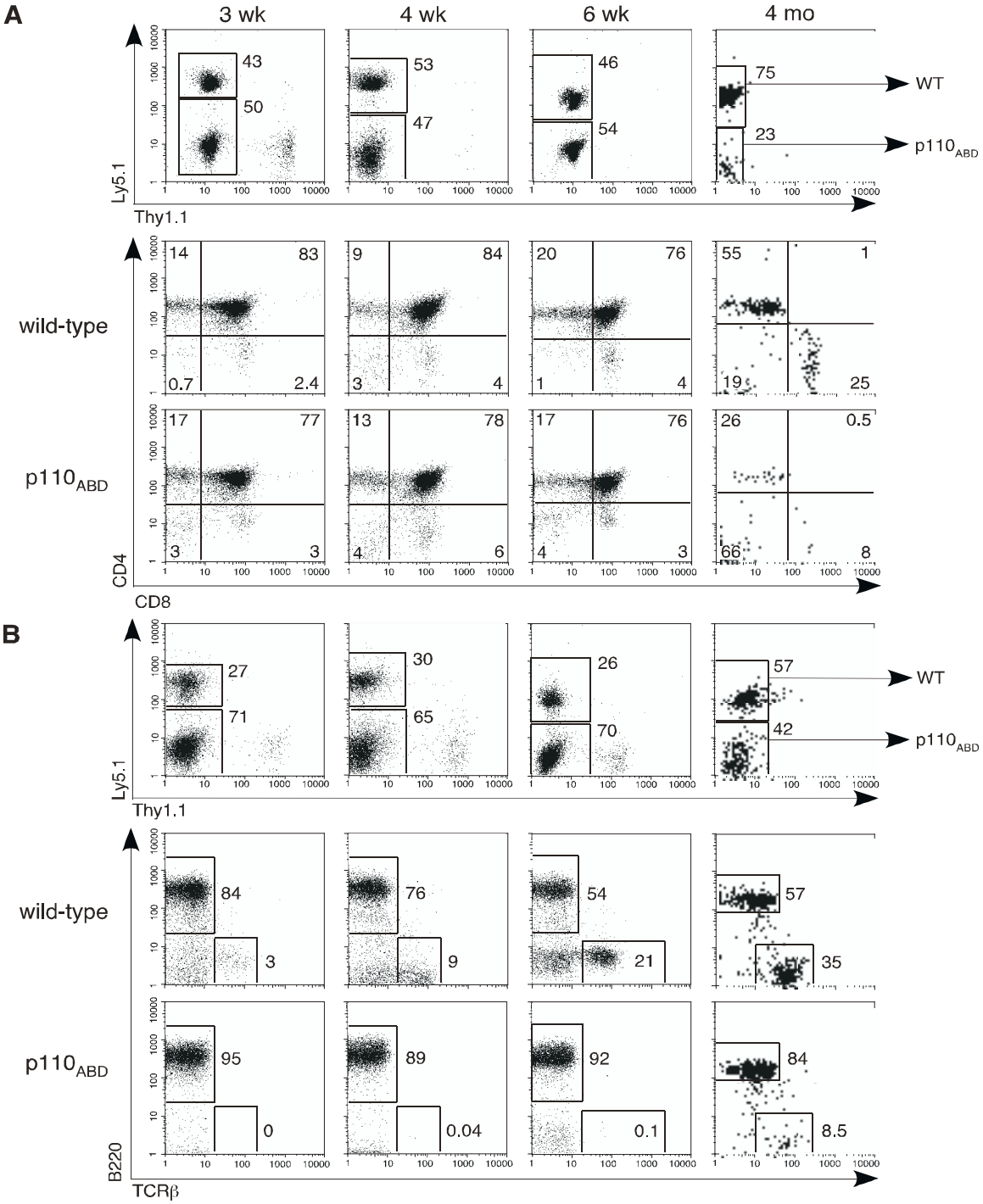

Figure 15. $p110_{ABD}$ thymocyte egress is delayed relative to WT in competitive adoptive transfers.

A. Thymic development of wild-type (Ly5.1 Thy1.2) and $p110_{ABD}$ (Ly5.2 Thy1.2)-derived cells is reconstituted by 3 weeks in lethally-irradiated recipients (Ly5.2 Thy1.1).

B. Although WT-derived T cells appear in the periphery of recipient mice by 3 weeks post-transfer, $p110_{ABD}$-derived T cells do not exit the thymus until after 6 weeks. Even at 4 months, T cells account for a smaller fraction of $p110_{ABD}$-derived cells than of nontransgenic-derived cells.

very few emigrants for two reasons. Firstly, homeostatic proliferation is induced by a paucity of T cells in the periphery, but wild-type cells successfully fill the T cell compartment in recipient animals prior to the appearance of $p110_{ABD}$-derived T cells. Thus any expansion of transgenic T cells is extremely minor. Secondly, the *lck* proximal promoter is downregulated following the completion of positive selection, and mature T cells exclusively use the distal promoter. When transgenic mice were made using the p1017 vector driving a lacZ reporter element, both RNA transcripts and reporter activity were undetectable in peripheral T cells (252). Thus $p110_{ABD}$-induced PI3K activity could not contribute to the signals that drive homeostatic proliferation. 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.

The emigration defect that we observe contradicts that described in $p65^{PI3K}$ mice and instead resembles that of $p110\gamma^{-/-}$ animals (81). $p65^{PI3K}$ expression increases the fraction of thymically-labeled $FITC^+$ or $BrdU^+$ CD4 T cells in the periphery. The potentiation of thymic exit is restricted to the CD4 lineage, however, as both wild-type and $p65^{PI3K}$ spleens contain similar numbers of labeled CD8 T cells. p110γ-deficiency impairs the emigration of cells from both lineages, by contrast. Intriguingly, $p65^{PI3K}$ expression in $p110\gamma^{-/-}$ mice restores thymic exit, and the numbers of $FITC^+$ or $BrdU^+$ splenic T cells of both lineages are similar to those in $p110\gamma^{+/-}$ mice. The $p65^{PI3K}$-mediated enhancement of CD4 T cell exit correlates with the enhancement of CD4 SP differentiation, with the sum total result of fewer CD4 SP thymocytes in adult animals compared to wild-type. It is therefore tempting to speculate that $p65^{PI3K}$ expression couples the induction of exit to the completion of positive selection. The biochemical basis of these effects and their differences will be discussed further below.

4.3 Chemokine functions in p110$_{ABD}$ mice

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 G_α subunits, as PTX expression in the thymus blocks migration across the CMJ and into the periphery, resulting in the accumulation of functionally mature SP cells in the cortex (107). The activation of PI3K molecules downstream of GPCR has been demonstrated in multiple systems, including chemokine receptors in T lymphocytes. It is thus likely that the p110$_{ABD}$ transgene is modulating chemokine signals that regulate thymic exit.

Despite the clear role of PTX-sensitive (and likely chemokine-mediated) signals in thymic egress, the molecular identity of the receptors involved is not clear. Two main candidates have been proposed: CCR7 and S1P$_1$. CCR7 expression is upregulated in post-selection thymocytes by combined Ras/Erk and Ca^{++} signals, and this is accompanied by the acquisition of responsiveness to CCL19 and CCL21 in *in vitro* chemotaxis assays (97, 98). However CCR7$^{-/-}$ mice do not have a clear thymic exit phenotype (108). Likewise, the spontaneous mutant "*p*aucity of *l*ymph node *T* cells" (*plt*/*plt*), which lacks all hematopoietic CCL19 and CCL21 expression, does not display any thymic phenotype that suggests a defect in the release of mature, naive T cells from the thymus (109, 110, 253). Studies of CCR7$^{-/-}$ and *plt*/*plt* mice suggest that CCR7 may instead be important for proper T cell homing in the periphery. CCR7$^{-/-}$ lymphocytes cannot colonize lymph nodes efficiently upon transfer, nor can transferred wild-type cells home properly in *plt*/*plt* animals (108, 254). PTX transgenic thymocytes likewise fail to properly enter peripheral lymph nodes when transferred into irradiated animals (107). It therefore seems likely that thymocyte

exit and peripheral lymph node entry, while both GPCR-mediated events, are distinct mechanisms and that CCR7 is primarily used in the latter process.

More recently, attention has shifted to the S1P receptors (reviewed in (255)). $S1P_1$ expression and S1P chemotactic responsiveness are also upregulated following positive selection (114, 116). $S1P_1$ agonist treatment induces the maturation of SP thymocytes to a $CD62L^{hi}$ stage but prevents exit from the thymus (115). On the other hand, $S1P_1^{-/-}$ SP thymocytes are predominantly $CD62L^{hi}$ and HSA^{lo} but fail to fully downregulate CD69 expression, a final maturation step that appears to be required for thymic egress since $S1P_1$-deficient thymocytes exhibit a profound defect in emigration (114, 116). T cell homing to peripheral lymphoid tissues is normal, as $S1P_1^{-/-}$ thymocytes transferred intravenously colonize the lymph nodes and peyers patches of wild-type host animals (114). In view of the PTX transgenic experiments and the effects of CCR7/CCL19/CCL21 loss, $S1P_1$ is an attractive candidate for the regulator of thymic exit.

We assessed the expression of several developmentally-regulated GPCR in sorted $CD3^{hi}$ thymocytes, to verify that our $p110_{ABD}$ emigration defect was not simply due to such changes. We used quantitative real-time RT-PCR to evaluate expression of $S1P_1$ and CCR7, which should be restricted to late thymocytes. Indeed, when expression is normalized to the amount in total thymocytes, we find that both of these GPCR are highly upregulated in mature thymocyte populations (Fig. 16A). In agreement with the expression pattern described for $S1P_1$, we find that the late $CD3^{hi}$ HSA^{lo} SP express the highest amounts. On the other hand, we do not observe increased expression at successive stages, as DP $CD3^{hi}$ and SP $CD3^{hi}$ HSA^{hi} thymocytes express the same relative amount of $S1P_1$. We also examined a receptor that is expressed at all stages of thymocyte development, CCR9, and one that is restricted to early thymocyte subsets, CXCR4. Again, we observe that NLC and $p110_{ABD}$ thymocyte subsets express roughly equivalent amounts

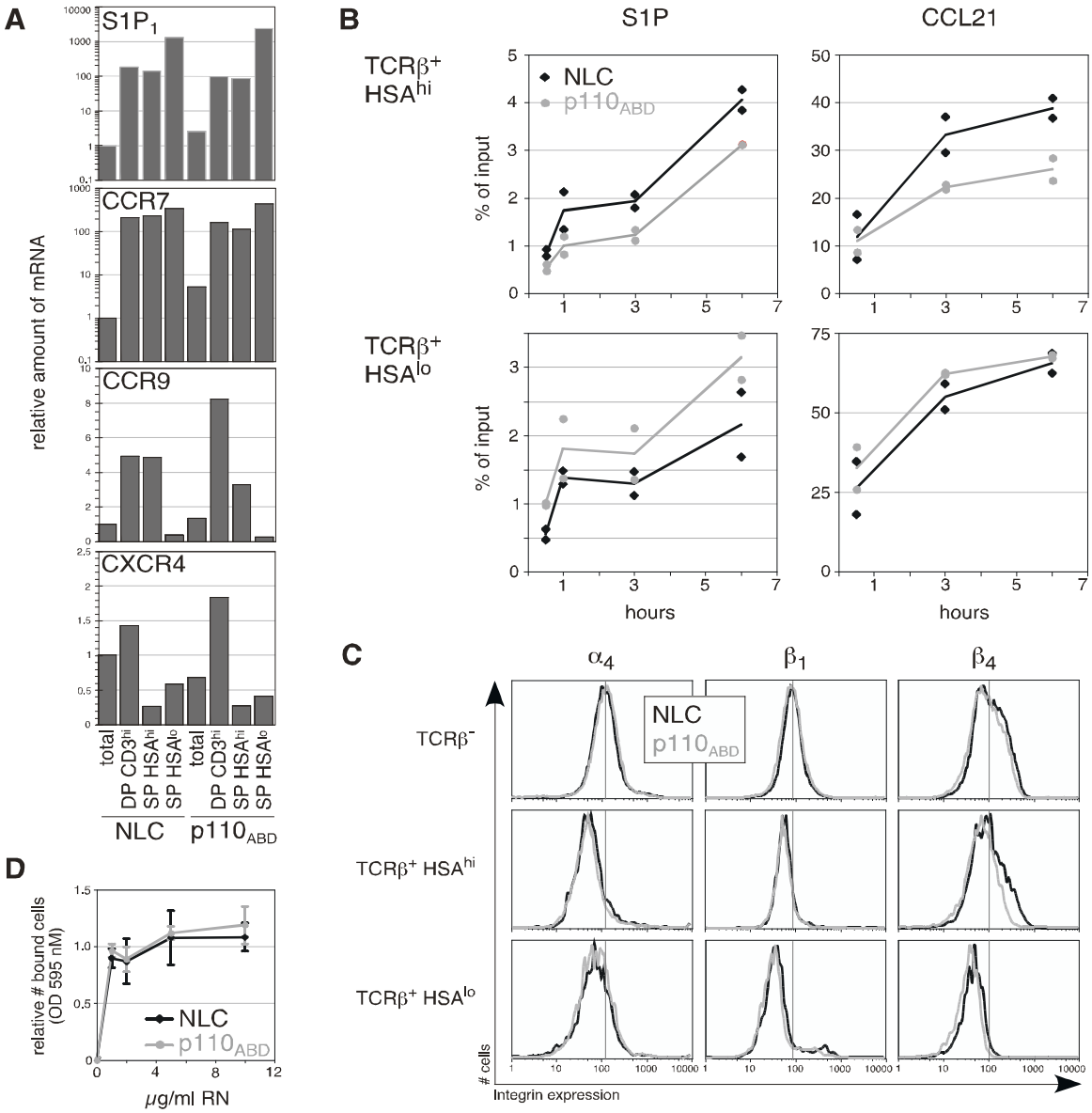

Figure 16. $p110_{ABD}$ expression does not alter chemokine receptor expression or chemokine responses.

A. The expression levels of multiple GPCR were assessed by quantitative real-time RT-PCR. Note that $S1P_1$ and CCR7 are shown with a logarithmic scale. All transcripts were normalized to GADPH expression using the formula $2^{-\Delta\Delta C_T}$.

B. *In vitro* transwell chemotaxis assays reveal no changes in SP thymocyte responses to S1P or CCL21.

C. Both NLC and $p110_{ABD}$ thymocytes slightly downregulate the integrins α_4, β_1, and β_4 during maturation.

D. Total thymocyte suspensions from NLC and $p110_{ABD}$ mice were analysed for their ability to bind to Retronectin *in vitro*. Adhesion was quantitated by staining with crystal violet and measuring the absorbance at 595 nM. When purified $TCR\beta^+$ HSA^{lo} thymocytes were used, the number of bound cells was below the level of reproducible detection (<0.2 OD).

of these mRNAs (Fig. 16A). Although there appears to be some alteration in the pattern of CCR9 expression, the changes are less than two-fold (note that the Y axes of the CCR9 and CXCR4 graphs are linear, whereas the $S1P_1$ and CCR7 axes are logarithmic). We conclude that any modulation of chemokine responses by $p110_{ABD}$ is mediated by altered signal transduction mechanisms rather than by altered expression.

We also attempted to assess any potential changes in the chemotactic responses to possible emigration factors, namely via $S1P_1$ and CCR7. NLC and $p110_{ABD}$ thymocytes were subjected to transwell chemotaxis assays using S1P or CCL21 as a chemoattractant. The phenotype and number of responding cells was assessed by flow cytometry. The migrants in these assays are preferentially enriched for SP thymocytes as expected, and the % of input that we measured fit in the ranges previously described. However we were unable to detect any reproducible difference between NLC and $p110_{ABD}$ responses. One experiment of three is shown in Figure 15B: unfortunately, all three experiments gave sufficiently different results to make statistical analysis fruitless. When each experiment is examined independently, we observe no significant change induced by $p110_{ABD}$ expression. This can be seen in the very minor differences depicted in Figure 16B. This suggests that these GPCR are insensitive to alterations in PI3K activity, and that PI3K instead mediates the functions of some other regulator of thymocyte egress.

4.4 Integrin expression and function is unaltered by $p110_{ABD}$ expression

The lack of a correlation between our exit phenotype and the chemotactic responses to known egress candidates suggests that some other, as-yet unidentified GPCR is important in this process. We therefore examined some of the phenotypic and functional consequences of chemokine

signaling. One of the most striking responses to chemokine treatment is the upregulation of integrin function. This can be observed in the ability of CCL21 to induce lymphocyte arrest on ICAM-1-coated surfaces under flow conditions (256). Integrins are heterodimeric receptors composed of α and β subunits, of which there are several that dimerize in a variety of combinations. Importantly, integrin function can be enhanced by both antigenic and chemotactic activation via two distinct "inside-out" mechanisms (reviewed in (257)). The first of these is a conformational change in the extracellular domain that increases affinity for ligand. Secondly, the avidity of integrin binding can be enhanced by changes in lateral mobility. PI3K signaling appears to be important for the latter form of integrin activation in lymphocytes. For example, β_1 integrin-mediated cell adhesion can be enhanced by TCR signaling in a manner that is sensitive to PI3K inhibitors (258). CD3-induced increases of T cell binding to the β_1 ligand fibronectin are also inhibited by the expression of kinase-dead Itk mutants (259). In cells expressing a membrane-targeted Itk that constitutively localizes to glycolipid-enriched rafts, CD4 cross-linking is sufficient to increase β_1 integrin activation independently of TCR signaling (259). It has been suggested that Itk recruitment to these rafts allows subsequent cytoskeletal remodeling that enhances integrin clustering. This model is supported by the observation that clustering increases the adhesion of LFA-1 (the $\alpha_L\beta_2$ integrin dimer) to ICAM-1 without increasing its affinity (as measured by binding of soluble ICAM-1) (260). Chemokines may specifically increase affinity by a PI3K-independent pathway as well as enhancing PI3K-induced clustering (256). CXCL12, CCL19, and CCL21 each activate Wortmannin-sensitive LFA-1 binding to ICAM-1 (256). PI3K inhibitors also impair chemokine-triggered LFA-1 clustering in the cell membrane and can completely block the formation of polarized cell "caps" (256). Importantly, PI3K activity is required for LFA-1-mediated arrest of cells on surfaces presenting low levels of ICAM-1 under physiological flow strength. When the affinity changes induced by chemokines are examined by binding to soluble ligand, however, PI3K inhibitors exert no effect (256). These

results demonstrate that PI3K activity can enhance integrin functions downstream of the TCR and chemokine receptors by mobilizing the movement of these molecules through the cell membrane.

Integrin expression, like that of chemokine receptors, is developmentally regulated. Although LFA-1 ($\alpha_L\beta_2$) is almost universally expressed on all thymocyte subsets (261), other integrins display more restricted expression patterns. The β_1 integrin forms two principal dimers, with α_4 and α_5, which are referred to as VLA4 and VLA5, respectively. Two additional dimers, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ are less common. All β_1 integrin dimers are expressed most highly in DN thymocytes and are progressively downregulated during development (262, 263). This expression pattern correlates with the ability of thymocytes to bind the β_1 ligand fibronectin: isolation of thymocytes by adhesion to fibronectin enriches DN and DP subsets (264). By contrast, SP thymocytes fail to bind fibronectin-coated surfaces (264). α_6 can also dimerize with the β_4 integrin. The expression of β_4 resembles that of β_1 dimers, but the β_4 sequence is the most divergent of all integrins and includes a very long (1000 amino acid), unique intracellular domain (265). While this suggests that β_4 may exert unique signaling functions, this integrin is dispensable for thymocyte development (266). Other integrins are required for proper thymocyte development, however. Tacβ1 is a trans-dominant inhibitor that blocks the activity of β_1, β_2, and β_3 integrins. Transgenic Tacβ1 expression impairs thymocyte adhesion to fibronectin and consequently induces a partial block at the DN to DP transition (262). β_1 activity is not absolutely required for thymocyte development, however, since some SP do develop in these mice. The various patterns of expression of and requirement for integrins suggest that these molecules cooperate with chemokine signals in the thymus. Together they may ensure that thymocytes interact with appropriate stromal cell partners at different stages of development.

In fact, integrin and chemokine expression may be coupled in mature T lymphocytes. Multiple integrins (including α_4, β_1, and β_2) are expressed at higher levels on CCR7$^-$ memory T cells compared to those cells that are CCR7$^+$ (267). Intriguingly, this negative correlation matches the coordinated expression of these molecules on thymocytes. We examined the surface expression of several integrins that are downregulated during thymocyte development. Surface levels of α_4, β_1, and β_4 are all reduced to intermediate levels in mature thymocytes subsets (Fig. 16C), in agreement with previous results. p110$_{ABD}$ thymocyte subsets express the same levels of these integrins as NLC cells. Taken in conjunction with the GPCR RT-PCR data, from this we conclude that the developmental program of chemokine receptor and integrin expression is unaltered by p110$_{ABD}$ expression.

Given the role of PI3K described in integrin activation, we sought to determine if p110$_{ABD}$ expression altered the ability of thymocytes to bind to integrin ligands. Since TCRβ^- thymocytes exhibit the highest integrin function, this could be a signal that is normally induced by chemokine receptors in DN and DP cells to direct proper cell-cell associations required for efficient development. Inappropriate binding induced by p110$_{ABD}$ could thus retain mature thymocytes in the thymic stroma. Since β_1 activity can be regulated by PI3K and Itk in T cells, and since β_1 activity is normally downregulated during thymocyte development, we decided to examine β_1 function in p110$_{ABD}$ thymocytes. Furthermore, expression of the β_1 ligand fibronectin is highest in the medulla (268), and we observe proper SP thymocyte localization to this compartment (Fig. 13C). We thus assessed the ability of thymocytes to adhere to retronectin-coated surfaces *in vitro*. Retronectin is a recombinant fragment of human fibronectin with a higher affinity for integrins, and we observe that thymocytes bind better to surfaces coated with retronectin compared to fibronectin. We chose to use this ligand because the more robust response should allow a greater dynamic range of detection. Thymocytes were allowed to adhere to 96-well plates

coated with varying concentrations. After the removal of non-adherent cells, the efficiency of adhesion was quantitated by staining adherent cells with crystal violet and measuring the OD at 595 nM. We find that total thymocytes from NLC and $p110_{ABD}$ animals bind to retronectin with the same dose-dependence (Fig. 16D). However, these bound populations should largely be composed of DN and DP cells. We attempted to specifically assess the ability of purified SP thymocytes to adhere to retronectin, but both NLC and $p110_{ABD}$ SP cells failed to bind. The crystal violet staining in these tests was below the level of reproducible detection (<0.02 OD), and visual examination revealed virtually no bound cells. Although PI3K can enhance the function of β_1 integrins in mature T cells, $p110_{ABD}$-induced PI3K activity does not do so in SP thymocytes.

4.5 How does $p110_{ABD}$ inhibit thymic exit?

Studies of $p110\gamma^{-/-}$ animals have revealed a critically important role for the class I_B PI3K in the chemotactic responses of multiple haematopoietic cells. For example, *in vitro* chemotactic responses of neutrophils and macrophages are decreased in $p110\gamma^{-/-}$ mice compared to wild-type (175, 195). Furthermore, p110γ-deficiency impairs inflammatory responses *in vivo* by disrupting cell recruitment. Peritoneal accumulation of neutrophils in response to challenge with *Listeria monocytogenes* is halved in $p110\gamma^{-/-}$ mice compared to wild-type (175). Recruitment of neutrophils and macrophages in septic peritonitis responses is almost completely abrogated as well, and p110γ-deficient animals are subsequently unable to clear peritoneal bacteria (195). These effects correlate with the loss of Akt phosphorylation and activity in response to chemokine treatment (175, 195).

On the other hand, p110γ is largely dispensable in lymphocyte chemotaxis. Thus far, a thymocyte emigration defect has only been reported for one of the two $p110\gamma^{-/-}$ lines that have been described (81, 175). Even in those animals, thymic exit is merely impaired, not severely compromised as in the PTX transgenic mice. The migration of mature T cells was not explicitly examined in p110γ-null animals and the chemotactic abilitites of these cells must be inferred. $p110\gamma^{-/-}$ spleens contain half the number of CD4 T cells in $p110\gamma^{+/-}$ mice, but this may reflect the reduced size and output of the thymii of these mice since peripheral lymph node numbers are the same (155). $p110\gamma^{-/-}$ mice do show a reduced footpad swelling reaction compared to $p110\gamma^{+/-}$ when injected with LCMV (155). CTLs purified from the spleens of LCMV-challenged p110γ-deficient animals can lyse target cells *in vitro*, however, with the same efficiency as p110γ-competent CTLs. This suggests that the impaired footpad swelling response is due to inhibited T cell migration to the site of infection rather than to a defect in T cell activation. But these effects are all minor compared to the severe defects in myeloid lineage chemotaxis. PI3K usage by chemokine receptors may therefore be distinct in different lineages.

While examination of $p110\gamma^{-/-}$ mice suggests that Class I_A PI3Ks may also regulate lymphocyte chemotaxis, genetic studies in animals have not demonstrated any essential functions. This is probably due to the compensatory activity of different subunits discussed above, since thymocytes and T cells retain PI3K activity in mice lacking adaptor subunits. The most compelling evidence *in vivo* for the coordinate use of both class I_A and I_B PI3Ks in lymphocyte chemotaxis is that of $p110\gamma^{-/-}$ mice expressing the activating $p65^{PI3K}$ transgene. $p65^{PI3K}$ expression is sufficient to restore normal thymic export in p110γ-null animals (81). On the other hand, a requirement for class I_A PI3K has only been demonstrated *in vitro* with cell line clones. Ward *et al*. have established Tet-Off Jurkat clones in which the expression of dominant negative PI3K

mutants can be regulated in a dose-dependent fashion (188). Δp85 expression can inhibit CXCL12-induced chemotaxis of these cells, albeit to a smaller degree than a kinase-dead p110γ mutant (188). To date no dominant-negative class I_A PI3K mice have been described. Unfortunately, our own attempts to generate Δp85 mice using the p1017 vector were unsuccessful. Although class I_A PI3K clearly can influence chemotactic responses in lymphocytes, a requirement for p85/p110 dimers remains to be conclusively proven.

Chemokine receptor triggering induces the activity of multiple PI3K effectors in lymphocytes, but the roles of these signals are only slowly emerging. The only T cell-specific chemokine signal thus far described that does not induce Akt phosphorylation and activity is CCL20 (LARC/MIP3α) and its receptor CCR6 (269). Although Akt activation is reported, the importance or requirement for this PI3K effector has not been demonstrated in lymphocytes. Rather the chemotactic requirement for Akt has been extensively studied in *Dictyostelium*, which will be discussed in detail below.

Recent reports do suggest a chemotactic requirement for the T cell-specific and PI3K-dependent Tec kinase Itk. Calcium flux is rapidly activated in response to most T cell-activating chemokines, including CXCL12, CCL19, CCL21, CCL25, CCL2 (MCP1), CCL5 (RANTES), and IL8 (269-274). Whether chemokine-induced increases of intracellular calcium are mediated by Itk remains unclear, however. CXCL12-induced Ca^{++} fluxes in T lymphoblasts are insensitive to Wortmannin (184). On the other hand, Jurkat Ca^{++} flux responses to CXCL12 are reduced by expression of a kinase-dead Itk and by knockdown of Itk levels via siRNA (275). The answer may lie in the usage of multiple PLC isoforms by chemokine receptors. For example, calcium fluxes in NK cells stimulated with CXCL12 can be severely impaired by anti-PLCβ but not anti-PLCγ antibodies (276). Furthermore, chemokine-induced Ca^{++} influx is completely abrogated in

neutrophils lacking both PLCβ2 and PLCβ3 isoforms (277). Clearly the calcium flux responses to chemokines, and potential roles for PI3K in their regulation, remain a poorly-understood field.

Itk may instead be required for cytoskeletal remodeling in response to chemokine receptor triggering, rather than calcium influx. This was first suggested by studies of CXCL12-induced Jurkat polarization (275). The polymerization of actin in response to CXCL12 is decreased in Jurkat cells expressing a kinase-dead Itk mutant (275). This effect can be traced to impaired activation of Rac and Cdc42 when the GTP-binding of these factors is assessed (275). These results are complemented by the observation that Itk-null T cells and thymocytes exhibit impaired migration to CXCL12 *in vitro* (278). Furthermore, intranasal administration of CXCL12 fails to recruit $Itk^{-/-}$ T cells *in vivo*, whereas wild-type cells rapidly accumulate in the lung (278). Note that PI3K- and Itk-mediated integrin activation appear to operate via integrin clustering in the membrane (discussed above). It is therefore tempting to speculate that Itk can contribute to chemotaxis by cytoskeletal remodeling that enhances cell-cell contact and lymphocyte rolling.

Recent work has demonstrated that the CDM family of DOCK proteins can contribute to Rac activation downstream of chemokine receptors. DOCK2 deficiency specifically impairs Rac activation by CXCL12, and lymphocyte migration is broadly defective in $DOCK2^{-/-}$ mice (279). In particular, $DOCK2^{-/-}$ DP and SP thymocytes fail to migrate in response to CXCL12 and CCL19, respectively (279). The regulation of DOCK2 is still unknown, but may involve PI3K. The related DOCK180 can bind PtdIns(3,4,5)P_3 *in vitro* despite lacking a conventional PH domain (280). Although a direct association with PtdIns(3,4,5)P_3 has not yet been described for DOCK2, an indirect association may be mediated via ELMO proteins. These scaffolding molecules are evolutionarily conserved and contain a PH domain critical for cytoskeletal reorganization (281). Whether Itk and DOCK2 cooperate downstream of PI3K to mediate actin

remodeling or whether they instead integrate separable GPCR signals remains to be determined.

How do we interpret the defect in $p110_{ABD}$ thymocyte egress in light of these results? Given the positive role of PI3K activity in chemotaxis discussed above, we should expect to observe enhanced chemotactic responses in $p110_{ABD}$ thymocytes. In order to make sense of our results, we must consider work performed in the slime mold *Dictyostelium* by Firtel, *et al.*. *Dictyostelium* cells are large, contain ample cytoplasm, have a dynamic cytoskeleton, and polarize and move rapidly in response to chemoattractants: they thus provide an excellent system in which study and visualize chemotaxis. Furthermore, abundant evidence indicates that PI3K and Akt are critical regulators of *Dictyostelium* chemotaxis (reviewed in (282)). One of the most striking features of chemotactic responses is the ability of cells to detect very minor differences in chemokine concentration. These shallow extracellular gradients are translated into steep intracellular gradients of signaling molecules via the highly-controlled accumulation of PtdIns(3,4,5)P_3 at the leading edge of the cell (283). This polarization requires the coordinated function of PI3K at the leading edge and PTEN at the sides and rear (283). Consequently Akt is rapidly and transiently recruited to the leading edge of the cell (284). In resting cells, an Akt PH domain-GFP fusion protein is evenly distributed throughout the cytoplasm (284). Upon stimulation with chemoattractant, this protein translocates to the membrane within 3 seconds. Maximal accumulation is observed between 5 and 8 seconds, and translocation lasts no longer than 22 seconds. This rapid return to the cytoplasm is referred to as "adaptation," and it allows the cell to essentially "reset." 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. Expression of a myristoylated Akt results in uniform membrane distribution that resembles that observed when cells are uniformly bathed in chemoattractant (284). *Dictyostelium* cells expressing myrAkt consequently migrate more slowly in response to

chemoattractants (284). Efficient chemotaxis thus requires strictly regulated membrane localization of PI3K products and effectors.

We suspect that chemotaxis of mammalian cells also depends upon regulated spatial organization. This notion is supported by visualization of $p110\gamma^{-/-}$ neutrophil migration in Zygmond chambers: p110γ-null cells do not migrate in an oriented direction toward a chemoattractant but instead move in all directions (285). Furthermore, $p110\gamma^{-/-}$ neutrophils move at half the speed of wild-type cells (285). Lymphocytes also polarize in the presence of chemokine gradients. Stimulation of T lymphoblasts with CCL2 or CCL5 induces rapid clustering of CCR2 or CCR5, respectively, that is oriented toward the chemokine (91). T cells can specifically accumulate responding chemokine receptors at the leading edge of cell movement.

We therefore propose that $p110_{ABD}$ expression impairs thymocyte migration by interfering with the proper spatial organization of the cell membrane. By uncoupling endogenous p110 from adapter subunits, $p110_{ABD}$ induces unregulated accumulation of PtdIns(3,4,5)P_3 in the cell membrane. This can be observed by the constitutive activation of Akt in $p110_{ABD}$ thymocytes. We suspect that this results in uniform membrane distribution of active Akt that in turn inhibits the ability of the thymocyte to properly respond to chemotactic signals. This should be in contrast to the effects of $p65^{PI3K}$. Since $p65^{PI3K}$ contains most of its protein-protein interaction domains, it should still couple endogenous p110 subunits to stimulating receptors with appropriate spatial organization. Unfortunately, unlike *Dictyostelium*, thymocytes are extremely small and contain very little cytoplasm. We have attempted to visualize membrane distribution of PtdIns(3,4,5)P_3 by retrovirally introducing an Akt PH-GFP fusion protein. But even at the highest magnification we could not discriminate between membrane and cytoplasmic localization. Attempts to visualize $p110_{ABD}$ thymocyte movement and PH-GFP organization upon

chemokine application are currently precluded by experimental constraints.

These results indicate that one must be careful when correlating the biochemical activity of transgenes with the functional consequences. Spatial organization of signaling molecules is rapidly emerging as an important mode of regulation and must be considered. We conclude by proposing that $p110_{ABD}$ expression disrupts the ability of thymocytes to respond to emigration signals in a properly oriented fashion.

5. Conclusions

Class I PI3Ks are involved in both the regulation of mature T and B cell function and the development of T and B cells (286, 287). Studies using PI3K knockout mice have demonstrated that alterations in both the adaptor (p85α, p55α p50α) (288, 289) and catalytic (p110δ) subunits of PI3K (178), 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 ($p110_{ABD}$) in thymocytes, to analyze the role of PI3K in immature DP and mature SP thymocytes. The ability of our $p110_{ABD}$ construct to coimmunoprecipitate 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 $p110_{ABD}$ 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. We also observe an improved ability to mobilize Ca^{++} in response to sub-optimal concentrations of anti-CD3 antibodies. We attribute this to a potentiation of TCR-mediated Itk activation and subsequent PLCγ induction.

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 could be the result of different processes in the DP compartment: 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. The observed phenotype could also be the consequence of changes in SP behavior, such as enhanced proliferation or a defect in thymocyte emigration. Any of these mechanisms would agree with the role of PI3K described in other cell types and organisms.

An enhancement in survival would be consistent with the modification of DP survival reported in gagPKB and Akt1$^{-/-}$ mice (179, 290). However, in spite of elevated Akt activity, we did not find changes in Bad phosphorylation or Bcl-X_L levels in p110$_{ABD}$ thymocytes. Nor did resting p110$_{ABD}$ thymocytes exhibit increased NF-κB DNA-binding activity compared to NLC. When we specifically examined the survival of DP thymocytes *in vitro* in the absence of antigen or when cultured with a corticosteroid, we found that NLC and p110$_{ABD}$ cells behave identically. DP survival was likewise unaffected by the expression of the activating transgene p65^{PI3K}, in agreement with our results (81). Our observations suggest that Akt may play a minimal role downstream of the TCR in thymocytes.

The increased numbers of mature T cells in the thymus of p110$_{ABD}$ mice could also be due to improved positive selection. To test this possibility we analyzed positive selection of the AND Tg TCR in a weakly selecting background (H-2$^{b/d}$). Our results show that expression of p110$_{ABD}$ dramatically improved selection in the H-2$^{b/d}$ background, almost restoring that observed in an optimal selecting background, H-2$^{b/b}$. In order to discriminate emigration-related effects from improved positive selection effects, we designed a BrdU tracking experiment to assess the daily

generation of new SP thymocytes. We found that $p110_{ABD}$ expression does specifically improve positive selection, since a greater fraction of AND $p110_{ABD}$ SP thymocytes are freshly generated from a labeled cohort: more $BrdU^+$ cells differentiate to SP in AND $p110_{ABD}$ mice than in AND animals. By contrast, we were unable to detect any affects of $p110_{ABD}$ on negative selection, either *in vivo* or using an *in vitro* deletion assay.

Positive selection is broadly impaired in $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ mice and correlates with defective Ca^{++} flux responses to TCR cross-linking (85-87, 235). Since Rlk lacks a PH domain and is PI3K-independent, we are confident that PI3K regulates positive selection via Itk and Ca^{++} influx modulation. The maximal Ca^{++} response is not increased by $p110_{ABD}$ expression, 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 (72), are critical for negative selection, and negative selection is also disrupted by loss of Itk, 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. It is also 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*. However, overexpression in the thymus of low levels of constitutively active calcineurin, a calcium regulated phosphatase, results in improved positive selection without alterations in negative selection (73). The role of calcineurin in the regulation of Nur77 remains controversial, but there is evidence to suggest that calcineurin and Nur77 represent separate signaling pathways downstream of Ca^{++} (238).

The findings on PI3K, Itk, calcineurin, and Nur77 are interesting to note in the context of the

quantitative versus qualitative models for thymocyte selection. From the reported roles of these molecules, we can deduce that Ca^{++} influx constitutes a mechanism by which quantitative differences in signal intensity can be converted into the activation of qualitatively different pathways. We postulate that low-intensity signals can activate positive selection pathways (possibly calcineurin-related). By contrast, high-intensity signals provoke pathways that mediate negative selection which may include Nur77. Thus, the conversion of negative selection to positive selection observed in some $Itk^{-/-}$ Tg TCR mice is the consequence of impaired Ca^{++} flux. According to this interpretation, $p110_{ABD}$-induced PI3K activity is sufficient to lower the threshold required for positive selection but not for negative selection. Whether this mechanism conforms to the threshold or duration model remains unclear, however. Our observation that $p110_{ABD}$ specifically enhances the primary Ca^{++} influx seems to suggest that the threshold model most accurately represents the mechanism in play. On the other hand, the primary Ca^{++} influx appears normal $Itk^{-/-}$ and $Rlk^{-/-}Itk^{-/-}$ thymocytes; rather the defect lies in a reduction of the sustained levels of intracellular calcium, which argues instead for the duration model. It should be noted, however, that Schaeffer *et al.* observe a slower decrease in intracellular Ca^{++} following the initial peak in wild-type cells than we do.

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 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.

The accumulation of mature SP CD3^{hi} thymocytes that we observe in p110$_{ABD}$ mice appears to be a compounded effect of enhanced positive selection and delayed thymic exit. We observe delayed colonization of peripheral tissues by p110$_{ABD}$ T cells in neonatal mice. The delay is even more striking in adoptive transfer recipients, in which significant numbers of wild-type derived T cells appear in the periphery within 1 month but p110$_{ABD}$-derived T cells are still completely absent at 6 weeks. Even at 4 months post-transfer, the p110$_{ABD}$-derived cells have only achieved the representation observed of wild-type cells at 1 month. These results demonstrate that PI3K is an important component of the signaling machinery that controls thymic exit.

Previous experiments suggested that an activating PI3K transgene (p65^{PI3K}) increased the emigration of CD4, but not CD8, thymocytes to the periphery (81), in direct contradiction of our observations. It is unclear how these results can be reconciled, although maybe 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 versus high concentrations of at least one chemokine, CXCL12 (291), whose receptor (CXCR4) can activate Tec kinases in thymocytes and mature T lymphocytes (275, 278). Perhaps such a concentration-dependent "switch" also applies to the PI3K activity induced by the emigration signal. But we instead suspect that the difference lies in the subcellular localization of PI3K catalytic activity in p110$_{ABD}$ versus p65^{PI3K} cells.

Elegant studies using GFP fusion proteins have demonstrated that chemotactic responses of *Dictyostelium* cells are highly dependent upon subcellular organization. Chemotactic responses are characterized by the rapid recruitment of Akt specifically to the leading edge of the cell. Importantly, Akt is rapidly shuttled back to the cytoplasm. It is suggested that this spatially restricted accumulation and subsequent adaptation essentially resets the cell. This is required for the cell to properly respond to the next chemotactic signal. Cells that express myrAkt exhibit

constitutive Akt localization at the membrane on all surfaces and chemotactic responses are subsequently slower. These results suggest to us that $p110_{ABD}$ thymocytes are less responsive to emigration signals because the disregulation of endogenous p110 recruitment to stimulating receptors induces the constitutive and non-specific recruitment of Akt to the plasma membrane. By contrast, $p65^{PI3K}$ can associate with endogenous p110 and contains all N-terminal protein-protein association domains. Constitutive PI3K activity should thus be restricted to appropriate locations on the membrane of $p65^{PI3K}$ cells, thereby enhancing chemotactic responses. While these theories are all currently speculation, they provide a tempting model to explain the diverse roles of seemingly similar transgenes. Furthermore, they point to a previously unappreciated aspect of control of signal transduction pathways.

In summary, we have demonstrated that PI3K can exert important functions during T lymphocyte development that closely parallel those in B cell development. PI3K is involved in signals from both the TCR and GPCR in thymocytes, and can mediate differentiation and migration responses to stimuli.

Appendix: 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. The construct was cloned into the BamHI site of the p1017 vector (204), and the NotI fragment purified and injected into (C57Bl/6 x DBA/2J)F_1 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). Subsequent progeny were genotyped by dot blot using the hGH probe or by PCR with hGH-specific primers. The generation of dnRas, Erk^{sem}, OT-1, HY, and AND transgenic animals has been described previously (29, 35, 36, 46, 49). Erk^{sem} mice were the kind gift of Stephen M. Hedrick (UCSD). OT-1, HY, AND, Thy1.1, Ly5.1, and DBA/2J ice were all obtained from Jackson Laboratories (Bar Harbor, ME)

Flow cytometric analyses

Phospho-S6 and phospho-Erk intracellular staining was performed as follows: 10^6 thymocytes were stimulated in a volume of 300 μL. At conclusion of stimulation, 40 μL of 16% paraformaldehyde was added directly. Samples were fixed for 10 minutes at 37°C, and then 1.8 mL of ice-cold methanol was rapidly added while votexing. Cells were incubated on ice for 30 minutes then washed once with PBS containing 4% FCS. Incubation with antibodies in 100 μL of PBS/FCS was performed for 20 minutes at room temperature (RT). Cells were washed thoroughly with vortexing to ensure removal of unbound antibody before analysis. Primary antibodies were obtained from Cell Signaling Technology (Beverly, MA): phospho-S6 Ribosomal protein (Ser235/236) cat. no. 2211; phospho-p44/42 MAPK (Thr202/Ty2204) E10 monoclonal cat. no. 9106. phospho-S6 was detected with PE-conjugated Donkey anti-Rabbit IgG

(Jackson ImmunoResearch cat. no. 711-116-152; West Grove, PA). phospho-Erk was detected by a two step amplification using biotin-conjugated Donkey anti-Mouse IgG (Jackson ImmunoResearch cat. no. 715-066-151) and Stretpavidin-PE (BD Pharmingen; San Diego, CA).

For Ca^{++} flux analyses, primary thymocytes (10^6/mL) were labeled with Indo-1 (2μg/mL in HBSS + 1% FBS; Molecular Probes; Eugene, OR) for 30 minutes at 37°C. Cells were centrifuged and stained with azide-free anti-CD8 FITC and anti-CD4 PE for 20 minutes at room temperature. Samples were then diluted to 5 x 10^5 cells/mL and incubated for ≥ 5 minutes at 37°C before analysis. Anti-CD3 antibody (2C11; BD Pharmingen) was added after 30 seconds, and Goat-anti-Hamster IgG (Jackson ImmunoResearch cat. no. 127-005-099) added at 5 minutes. Maximal Ca^{++} flux was measured following stimulation with 1-2 μg/mL Ionomycin (Sigma cat. no. I0634; St. Louis, MO), and calibrations were performed with the Calcium Calibration Buffer Kit #1 (Molecular Probes cat. no. C3008). The pre- and post-stimulation Ca^{++} levels corresponded with those previously published as determined by comparison to the calibration standard (292).

Bcl-2 intracellular staining was performed according to manufacturer's directions with the Cytofix/Cytoperm kit (BD Pharmingen cat. no. 2075KK). Briefly, freshly isolated thymocytes ($2x10^6$) were suspended in 200 μL Cytofix/Cytoperm and incubated on ice for 30 minutes. After washing with PBS containing 1% FCS and 0.01% NaAzide, cells were resuspended 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 minutes, then washed twice with Perm/Wash and resuspended in PBS/FCS/azide for analysis.

For simultaneous surface- and DNA content-staining, samples were first surface stained with FITC- and PE-conjugated antibodies. After thorough washing with PBS, cells were resuspended

in 875 μL PBS containing 0.1% Na Azide. Samples were vortexed immediately after addition of 125 μL 2%. Samples were fixed for 1 hour on ice, centrifuged, and all liquid aspirated. Cells were then permeabilized in 1 mL PBS containing 0.05% Tween 20 by incubation for 15 minutes at 37°C. Samples were washed thoroughly with PBS/azide and resuspended in 1mL 7AAD (25 μg/ml in PBS/azide; Molecular Probes cat. no. A1410). Samples were incubated on ice at least 30 minutes, washed, and then analyzed within 2 hours.

All surface antibodies were obtained from BD Pharmingen or eBioscience (San Diego, CA) and were used interchangably. Analyses were performed using a Beckton-Dickinson FACSCalibur or a Beckton-Dickinson FACScan, with the exception of Ca^{++} flux studies, which were performed on a BD FACSVantage. Cell sorting for GPCR RT-PCR was also performed on the FACSVantage. All other cell sorting was performed on MACS magnetic columns (Miltenyi Biotec, Auburn, CA), using biotinylated antibodies and Streptavidin Microbeads (Miltenyi Biotec). Analysis of flow cytometry data was performed using FlowJo (TreeStar Software, Ashland, OR) or CellQuest (Beckton Dickinson)

Western blotting

Western blots were performed as described (45). The following primary antibodies were used: rabbit anti-Bad C-20, mouse anti-Bcl-X_L H-5, rabbit anti-p85α Z-8, rabbit anti-p110α H-201, goat anti-p110α C-17, rabbit anti-p110δ H-219 (Santa Cruz Biotechnology, Santa Cruz, CA), sheep anti-Bad phospho-Ser136 #06-799, rabbit anti-p85α #06-195 (Upstate), and rabbit anti-MEK1/2 #9122 (Cell Signaling Technology).

Akt kinase assay

Akt "*in vitro*" kinase assays were performed using immunoprecipitates with an anti-Akt antibody

(Stressgen cat. no. KAP-PK004E; Victoria, BC Canada) or sepharose-conjugated anti-Akt (N-19, Santa Cruz Biotechnology). $2x10^7$ cells were stimulated on plates either directly coated with anti-CD3 antibodies (2C11) or coated with streptavidin (Pierce cat. no. 21122, Rockford, IL) and then biotinylated anti-CD3 (BD Pharmingen). After washing with cold PBS, stuimulated cells were resuspended in 200 μL Buffer A (50 mM TrisCl pH 7.5, 50 mM NaF, 5 mM Na pyrophosphate, 1mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 0.1% (w/v) Triton X-100, 0.1% (w/v) βME) containing 1 mM Mycrocystin-LR (BIOMOL cat. no. EI-193; Plymouth Meeting, PA). Samples were lysed for 5 minutes on ice, centrifuged at top speed 5 minutes, and then Akt was immunoprecipitated from the supernatant. Immunoprecipitates were washed twice with Buffer A + 0.5 M NaCl and once with Assay Dilution Buffer (25 mM β-glycerophosphate pH 7, 20 mM MOPS pH 7.2, 1 mM Na_3VO_4, 1 mM DTT). To the aspirated pellet was added: 10 μL Assay Dilution Buffer, 10 μL myelin basic protein (0.1 μg/mL; GibcoBRL cat. no. 13228-010; Gaithersburg, MD), and 10 μL [γ^{32}P] ATP (1 μCi/μL in 75 mM $MgCl_2$, 500 μM ATP). The reaction was performed for 15 minutes at 30°C and then stopped by the addition of SDS loading buffer. Samples were separated on a 12% SDS PAGE gel and either stained with Coomassie Blue and dried or transferred to nitrocellulose.

NF-kB gel shift analyses

Nuclei were purified by first preparing cytoplasmic extracts: 10^7 thymocytes were resuspended in 100 μL CE buffer (10 mM HEPES-KOH pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM AEBSF) + 0.1% NP40. Cells were lysed for 2 minutes on ice and then nuclei pelleted by microcentrifugation at 4000 rpm. Nuclei were lysed in 20 μL NE buffer (250 mM Tris pH 7.5, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM AEBSF) by repeated freeze-thawing in dry ice/95% EtOH and an ice water bath. Lysates were cleared by centrifugation at 13000 rpm for 10 minutes, quantitated with the Micro BCA* Protein Assay kit (Pierce cat. no. 23235), and then

adjusted to 1 μg/mL with NE buffer. The Igκ hairpin probe fragment was a generous gift of Alexander Hoffman and David Baltimore (CIT). The probe was radiolabeled with T4 kinase, and then probe (0.5 μL) and nuclear protein (2.5 μL) were incubated together for 30 minutes at RT in Binding Buffer (10 mM TrisCl pH 7.5, 50 mM NaCl, 10% glycerol, 1% NP40, 1 mM EDTA, 0.1 mg/mL poly(dIdC) (Pharmacia cat. no. 27-7880-02; Piscataway, NJ)). Samples were directly run on a 5% acrylamide gel (30:0.8/TGE/5% glycerol) for 2 hours at 150V. Gels were vaccuum-dried and exposed to phosphoscreens.

Ribonuclease protection assay

RNA was extracted from thymocytes either with TRIzol reagent (Invitrogen; Carlsbad, CA) or according to the single-step extraction protocol in Current Protocols in Molecular Biology (John Wiley & Sons, Inc.). All subsequent steps were performed according to manufacturer's instructions with the mAPO-2 probe template set (cat. no. 45354P), RiboQuant In Vitro Transcription kit (cat. no. 45004K), and RiboQuant RPA kit (cat. no. 45014K), all from BD Pharmingen.

In Vitro survival assays

10^6 thymocytes were aliquoted per well in a 96 well plate. For antigen-independent assays, each well was resuspended in 100 μL of DMEM complete with 10% FCS and βME. Plates were incubated at 37°C in 5% CO_2 for varying times and then analysed by flow cytometry. Viable DP thymocytes were determined by forward and side scatter. For Dexamethasone sensitivity assays, each well was resuspended in 100 μL DMEM complete/FCS/βME containing 0, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M Dexamethasone (Sigma cat. no. D1756). Plates were incubated for 12 hours 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.

BrdU labeling experiments

BrdU (Sigma cat. no. B5002) was supplied one of two ways: for DP steady state analysis, BrdU was provided continuously in drinking water at 0.8mg/ml until analysis; for pulse-chase analysis of SP differentiation in AND H-$2^{b/d}$ animals, BrdU was administered in 2 intra-peritoneal injections, each 1 mg in 200 μL PBS, 3 hours apart. BrdU staining was performed in duplicate as follows: cells were stained for surface antigens as usual, washed with PBS, and resuspended in 500 μL of 0.15 M NaCl. 1.2 mL of 95% EtOH at -20°C was added dropwise, and the cells incubated for 30 minutes on ice. Following a wash with PBS, the cells were resuspended in 1 mL of 1% paraformaldehyde/0.01% Tween-20 in PBS and incubated overnight at 4°C. The cells were then centrifuged and directly resuspended in 1 mL of DNase I solution (50 Kunitz units/mL in 0.15 M NaCl, 4.2 mM $MgCl_2$, 10 μM HCl; Sigma cat. no. D4527). After incubation for 30 minutes at 37°C, the samples were washed and stained with FITC anti-BrdU or isotype control antibody (BD Pharmingen).

OT-1 deletion assays

In vitro deletion assays were performed with a protocol adapted from that described previously (45). EL4 cells were irradiated with 3000 R and plated at 5 x 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-1 or OT-1 $p110_{ABD}$ thymocytes were added per well to a final volume of 200 μL and incubated for 18 hours at 37°C. Samples were then analyzed by flow cytometry as described for survival assays above.

Microscopy

Freshly-dissected thymic lobes were imbedded in Tissue-Tek OCT medium (Sakura Finetek cat. no. 4583; Torrance, CA) and flash-frozen on dry ice. Samples were stored at -80°C until

sectioning.

For Giemsa staining, 10 μm sections were fixed for 5 minutes in methanol. After rinsing thoroughly in water to remove remaining OCT, slides were immersed in methanol for 3 minutes twice. Jenners stain (1 g in 500 mL methanol, diluted 1:1 with water) was applied for 15 minutes, Giemsa stain (0.005% in water) for 5 minutes, and then slides were rinsed briefly in 95% and 100% ethanol. Coverslips were mounted with Permount (Fisher).

For immunoflourescent staining, 8 μm sections were fixed in methanol for 3 minutes and then washed in PBS with 1% FCS for 10 minutes 3x. CD8-FITC and CD4-PE antibodies (BD Pharmingen) were diluted in PBS/FCS and 100 μl applied to each section. Slides were incubated at RT in a humid chamber for 1 hour. After washing twice in PBS/FCS and twice in PBS, coverslips were mounted with Fluoromount-G (Southern Biotech cat. no. 0100-01; Birmingham, AL). Immunoflourescence was visualized using a Zeiss LSM Pascal inverted laser scanning microscope in the Caltech Biological Imaging Center. Data collection and analysis was performed using the v3.2 SP 2 software.

Competitive adoptive bone marrow transfer

Bone marrow from C57Bl/6 Ly5.1 and $p110_{ABD}$ mice were depleted of $CD3^+$ cells by MACS (Miltenyi Biotech), mixed at a 1:1 ratio, and injected into recipient Thy1.1 mice that had been lethally irradiated with 2 1200 R doses 3-4 hours apart. Mice were maintained on oral antibiotics from 3 days prior to transfer until time of analysis, at which point animals were sacrificed and the thymii, spleens, and mesenteric lymph nodes analyzed by flow cytometry.

Quantitative RT-PCR

Thymocyte subsets for RT-PCR were purified by FACS, and RNA prepared with the Ambion RNAqueous kit (cat. no. 1912; Austin, TX) according to manufacturer's instructions.

Contaminating genomic DNA was removed with the Ambion DNA-free kit (cat. no. 1906) and cDNA then synthesized with SuperScript II RT (Invitrogen cat. no. 18064-022). Real-time PCR was performed in triplicate with the SYBR Green system (Applied Biosystems cat. no. 4309155; Foster City, CA) on a ABI Prism 7700 machine. The following primers were used:

GADPH forward (189) 5'-ACTCCACTCACGGCAAATTCA-3'

GADPH reverse (309) 5'-GCCTCACCCCATTTGATGTT-3'

$S1P_1$ forward 5'-GTGTAGACCCAGAGTAATGCG-3'

$S1P_1$ reverse 5'-AGCTTTTCCTTGGCTGGAGAG-3'

CCR7 forward 5'-CAGCCTTCCTCTCTCATTTCTACA-3'

CCR7 reverse 5'-ACCACCAGCACGTTTTTCCT-3'

CCR9 forward 5'-TGGTCAATGGATGTTCCAGA-3'

CCR9 reverse 5'-TGCACATGATGAGAAGCACA

CXCR4 forward 5'-GAAACTGCTGGCTGAAAAGG-3'

CXCR4 reverse 5'-CTGTCATCCCCCTGACTGAT-3'

(The CCR7 and $S1P_1$ primers are those originally described in (114)). Quantitation of mRNA levels was performed according to manufacturer's instructions using the formula $2^{-\Delta\Delta C}T$.

Transwell chemotaxis assays

In vitro chemotaxis assays were performed as previously described (114). S1P was from Sigma. CCL21 was obtained from R&D Systems (cat. no. 457-6C; Minneapolis, MN) or PeproTech (cat. no. 250-13; Rocky Hill, NJ): both were used interchangeably. Chemokine dilutions were prepared in Migration Media (RPMI with 0.5% BSA, Pen/Strep, 10 mM HEPES). 600 μL was aliquoted per well in a 24 well plate and the plate transferred to a 37°C incubator to let equilibrate while preparing thymocytes. Freshly isolated thymocytes were resuspended in Migration Media at 10^7 per mL and allowed to pre-warm at 37°C for 10 minutes. Transwell filters (6.5 mm

Polycarbonate membrane, 5 μm pore size; Corning cat. no. 93421; Acton, MA) were inserted and 100 μL of thymocytes (10^6) aliquoted per chamber in duplicate. Plates were then incubated at 37°C in 5% CO_2. To harvest migrants, the insert was removed by drawing it along the side of the well to allow any medium on the underside to run back into the well. Cells were then collected and stained for flow cytometry analysis. The number of migrants was determined by collecting the entire sample during analysis.

Adhesion assays

Retronectin-coated plates were prepared by diluting retronectin in PBS pH 8, aliquoting 100 μL per well, and incubating for 2 hours at 37°C. Freshly isolated thymocytes were resuspended in warm Tyrodes solution with 10% FCS (+ Na Pyruvate, Pen/Strep, Non-essential amino acids, HEPES, 50 nM βME) at 10^7/mL. 100 μL/10^6 cells were aliquoted per well in triplicate and allowed to bind for 45 minutes at 37°C in 5% CO_2. After PBS (100 μL) was added to each well, the plate was inverted and gently centrifuged for 5 minutes at 100 g/700 rpm. All remaining liquid was aspirated and the adherent cells fixed in 70% EtOH for 20 mintes at RT. The plate was stained with 0.1% crystal violet in water for 5 minutes at RT and then rinsed briefly with water to remove unbound stain. Stained samples were resuspended in 0.2% Triton X-100 by incubation at RT overnight and then quantitated by measuring the OD at 595 nM. To specifically assess SP adhesion, pooled thymocytes were depleted of HSA^{hi} and $NK1.1^{hi}$ by MACS on LD^+ columns (anti-NK1.1 was included to ensure removal of NKT cells which are HSA^- (293)).

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