The Loss of PTEN Allows TCR $\alpha\beta$ Lineage Thymocytes to Bypass IL-7 and Pre-TCR–mediated Signaling

Thijs J. Hagenbeek,1,4 Marianne Naspetti,1 Fabrice Malergue,2 Fabien Garçon,5 Jacques A. Nunès,5 Kitty B.J.M. Cleutjens,6 Jan Trapman,6 Paul Krimpenfort,3 and Hergen Spits1,4

1Department of Immunology, 2Department of Cell Biology, and 3Department of Molecular Genetics, Netherlands Cancer Institute, 1066 CX Amsterdam, Netherlands
4Department of Cell Biology and Histology, University of Amsterdam, Academic Medical Center, 1105 AZ Amsterdam, Netherlands
5Institut National de la Sante et de la Recherche Medicale UMR 599, Institut de Recherches sur le Cancer de Marseille, 13009 Marseille, France
6Department of Pathology, Erasmus Medical Centre, 3000 DR Rotterdam, Netherlands

Abstract

The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) negatively regulates cell survival and proliferation mediated by phosphoinositol 3 kinases. We have explored the role of the phosphoinositol(3,4,5)$P_3$-phosphatase PTEN in T cell development by analyzing mice with a T cell–specific deletion of PTEN. $Pten^{fl/fl}$Lck-Cre mice developed thymic lymphomas, but before the onset of tumors, they showed normal thymic cellularity. To reveal a regulatory role of PTEN in proliferation of developing T cells we have crossed PTEN-deficient mice with mice deficient for interleukin (IL)-7 receptor and pre–T cell receptor (TCR) signaling. Analysis of mice deficient for $Pten$ and $CD3\gamma$; $Pten$ and $\gamma$; or $Pten$, $\gamma$, and $Rag2$ revealed that deletion of PTEN can substitute for both IL-7 and pre-TCR signals. These double- and triple-deficient mice all develop normal levels of CD4$^-$CD8$^-$ double negative and double positive thymocytes. These data indicate that PTEN is an important regulator of proliferation of developing T cells in the thymus.

Key words: PI-3K • thymus • Cre-LoxP • IL-7 receptor • pre–T cell receptor

Introduction

T cell development proceeds through various well-defined transitional cellular stages. T cell progenitors are negative for CD4, CD8, and CD3 and can be subdivided in four subpopulations on the basis of CD44 (Pgp-1) and CD25 (IL-2 receptor $\alpha$-chain) surface expression (1). The most primitive of these CD4$^-$CD8$^-$ (double negative [DN]) express CD44 and are negative for CD25 (DN1). These cells differentiate further into the intermediate DN stages with the phenotypes CD44$^+$CD25$^-$ (DN2) and CD44$^+$CD25$^+$ (DN3). TCR$\beta$ rearrangements are initiated in the CD44$^+$CD25$^+$ DN2 stage. When these rearrangements are successful, the translated TCR$\beta$ protein forms a pre-TCR complex with the pT$\alpha$ chain and signals emanating from this receptor result in survival and proliferation of TCR$\beta$-expressing cells (2, 3). As a consequence of $\beta$-selection, the CD44$^+$CD25$^+$ cells lose CD25, acquire CD2 and CD5 (4), and rapidly differentiate through an intermediate CD4$^+$CD8$^+$, immature (TCRlow) single positive (ISP) stage, to the CD4$^+$CD8$^+$ double positive (DP) stage.

In the early stages of T cell development, these cells go through two waves of proliferation: one mediated by the cytokines IL–7 and stem cell factor and the other by triggering of the pre-TCR complex. IL-7 and stem cell factor control the proliferation of the two first stages, DN1 and DN2, and survival of the DN3 cells (5–7). Pre-TCR triggering induces a second wave of extensive proliferation of
pre–T cells. Recently, we documented that phosphatidylinositol 3 kinase (PI-3K) is involved in IL-7–mediated cell survival because PI-3K associates with the IL-7Rα chain and a dominant-negative mutant of the p85 chain strongly inhibited T cell development in a fetal thymic organ culture (8). PI-3K converts phosphatidylinositol-(4,5)-bisphosphate (PtdIns[4,5]P2) to phosphatidylinositol-(3,4,5)-triphosphate (PtdIns[3,4,5]P3), which can bind pleckstrin homology domain-containing intracellular enzymes, including phosphoinositide-dependent kinase-1 (PDK-1), Akt/protein kinase B (PKB), and TEC family kinases such as IL-2–inducible T cell kinase (Itk) in T cells and Bruton agammaglobulinemia tyrosine kinase in B cells. PDK-1 phosphorylates Akt/PKB, which seems to be an important player in the regulation of cell survival of thymocytes and mature T cells (9). Overexpression of a constitutive active mutant of Akt/PKB results in elevated levels of the antipapoptotic molecule Bcl-X<sub>L</sub> and enhanced NF-κB activation through accelerated degradation of the inhibitory molecule Itkβ in both thymocytes and peripheral T cells (9). The PI-3K–Akt signal transduction pathway is counteracted by the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), whose lipid phosphatase activity is associated with tumor suppression (10). PTEN removes the D3 phosphate from PtdIns(3,4,5)P3 and negatively regulates survival signaling mediated by Akt/PKB and other downstream targets of PtdIns(3,4,5)P3 (for review see references 11–13). Thus, PTEN might be involved in the control of proliferation and survival in early T cells. An absence of PTEN leads to an increase of the basal levels of PtdIns(3,4,5)P3 and, hence, to a sustained signaling through mediators that are activated by PtdIns(3,4,5)P3.

Pten<sup>−/−</sup> null mutant knockout mice have been generated in other laboratories (14, 15). These mice die during early embryogenesis, precluding any assessment of the role of PTEN in the development of T cells. Pten heterozygous mice have increased spontaneous tumor incidence (15), lymphoid hyperplasia development, and display autoimmune disorders (16). The fact that some spontaneous tumors were of T cell origin suggested a role for PTEN in the development of T cells. PTEN-deficient mice have been described previously (20, 21). The conditional targeting vector and the floxed Pten<sup>flox/flox</sup> gene (<sup>Pten</sup>) were used for analysis as homozygous mutant, heterozygous mutant, and wild-type mice, respectively. The mice were maintained under specific pathogen-free conditions in the animal colony of the Netherlands Cancer Institute. CD3<sup>−γ−</sup> (20), γc-deficient (21) and Rag2<sup>−/−</sup>, γc–double deficient (22) mice were generated at the Netherlands Cancer Institute and have been described in detail previously. Pten<sup>flox/flox</sup>Lck-Cre mice were crossed with CD3<sup>−γ−</sup> or Rag2<sup>−/−</sup>, γc–double deficient mice to generate the various double and triple deficient mice.

**Materials and Methods**

**Generation of Mice.** The conditional targeting vector and the generation of mice carrying the Pten<sup>flox</sup> allele by blastocyst microinjection have been described previously (18). To generate T cell–specific Pten<sup>−/−</sup> mice, Pten<sup>flox/+</sup> mice were crossed with Lck-Cre transgenic mice (provided by Merck; reference 19). Offspring carrying Lck-Cre and the floxed Pten mutation on both alleles (Pten<sup>flox/flox</sup>Lck-Cre), Lck-Cre and the floxed Pten mutation on one allele (Pten<sup>flox/+Lck-Cre</sup>), and Lck-Cre and the wild-type Pten gene (Pten<sup>+/+</sup>Lck-Cre) were used for analysis as homozygous mutant, heterozygous mutant, and wild-type mice, respectively.

**PCR Analyses of Genotypes.** Genomic DNA was isolated from tail clippings and amplified by PCR following a standard protocol. Sense primer (5′-GCCCTACCCTAGTAAAAAGCAAG-3′) and antisense primer (5′-GGCAAGAATCTTGTTGTA-TAC-3′) were used to detect the Pten flox allele, and sense primer (5′-GCAAGTCACGGGTATCAAC-3′) and antisense primer (5′-GATGCAACAGGTGATGTTCT-3′) were used to detect the Lck-Cre transgene. Thermo-cycling conditions consisted of 31 cycles of 60 s at 94°C, 30 s at 58°C, and 40 s at 72°C. Reactions contained 200 ng of template DNA, 0.5 μM of primers, 100 μM dNTPs, 9% glycerol, 2.5 U Taq polymerase, 1.8 mM MgCl<sub>2</sub>, and PCR buffer (GIBCO BRL and Invitrogen) in a 25-μl volume. Amplified fragments of 230 bp (wild type), 280 bp (Pten<sup>flox/flox</sup>), and 350 bp (Cre), respectively, were obtained. Genotype analyses of CD3<sup>−γ−</sup>, γc-deficient, and Rag2<sup>−/−</sup>, γc–double deficient mice have been described previously (20, 21, 22).

**Immunoprecipitation and Immunoblot.** For analysis of Pten expression, 20 × 10<sup>5</sup> thymocytes from 4-wk-old Pten<sup>flox/flox</sup>Lck-Cre, Pten<sup>flox/+Lck-Cre</sup>, or wild-type mice were lysed in lysis buffer containing 1% NP-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 200 μM dithiothreitol, 200 μM MgCl<sub>2</sub>, and 5 μg/ml phosphatase inhibitor (Roche) in a lysis buffer. Lysates were processed and immunoprecipitated with specific antibodies against the phosphorylated form of the protein as described previously (18). Cells were lysed for 30 min on ice. After lysis, lysates were centrifuged at 2,000 × g for 5 min at 4°C to remove debris. Supernatants were then divided into 250-μl aliquots and incubated with affinity-purified antibodies at 4°C for 1 h. Immunoprecipitates were collected with agarose beads and washed three times with buffer containing 0.5% Triton X-100 for 10 min at 4°C. Immunoprecipitates were resolved by SDS-PAGE (10% acrylamide) and blotted with antibodies against the protein of interest as described previously (18). A anti-phospho-Akt/PKB (Ser473) and anti-Akt/PKB antibodies were purchased from Cell Signaling Technology.
NaCl, 5 mM EDTA, pH 8.0, and protease inhibitor cocktail (Roche Diagnostics). To be able to detect phosphorylated proteins, 50 mM NaF and 1 mM Na3VO4 were included in the lysis buffer. 30 μg of the soluble fractions was loaded on a 10% polyacrylamide gel in reducing conditions. After transfer on nitrocellulose membrane (Protran), the presence of PTEN protein was detected with the mouse monoclonal antibody specific for the COOH-terminal part of the protein (Santa Cruz Biotechnology, Inc.). To confirm equal loading, membranes were stripped using strip buffer (625 mM Tris, pH 6.8, 2% SDS, and 100 mM 2-mercapto-ethanol) and stained with antiactin antibody (Santa Cruz Biotechnology, Inc.). For analysis of Akt/PKB and Itk phosphorylation and Tec expression, thymocytes from 5- or 14-wk-old \( Pten^{+/+} \) mice or control (\( Pten^{+/+} \) or wild-type) mice were lysed in the aforementioned lysis buffer. Unstimulated or CD3-stimulated Jurkat T cells were included as controls. The anti–CD25 PE. For CD44, CD25 staining in CD3

Results

Phosphorylated Akt/PKB in the Thymus of \( Pten^{+/+} \) Lck-Cre Mice. T cell–specific \( Pten \)–deficient mice (\( Pten^{+/+} \) Lck-Cre mice) were generated by crossing \( Pten^{+/+} \) mice with \( Lck-Cre \) transgenic mice (19). \( Pten^{+/+} \) Lck-Cre mice were born alive and appeared healthy. Genomic PCR of tail DNA showed the amplification of a 280-bp band corresponding to the floxed allele (Fig. 1 A). The deletion of \( Pten \) exon 5 encoding the phosphatase domain of PTEN (26) in \( Pten^{+/+} \) Lck-Cre mice crossed with \( Lck-Cre \) mice was con-
The absence of PTEN in thymocytes results in an accelerated generation of DP thymocytes during ontogeny. (A) Percentages of double negative (DN; CD4+/CD8−), immature single positive (ISP; CD4+/CD8−), and double positive (DP; CD4+/CD8+) thymocytes of E16 old homozygote 
Ptenfloxflox/Lck-Cre (black bars, n = 3) or control (heterozygote or wild type; white bars, n = 4) embryos as determined by flow cytometry. (B) Flow cytometry of embryonic thymocytes. CD4CD8 staining of E16 old homozygote 
Ptenfloxflox/Lck-Cre (black bars, n = 3) or control (heterozygote or wild type) embryos. Numbers indicate percentages of gated populations. The total cell number mean is indicated for homozygote 
Ptenfloxflox/Lck-Cre (n = 3) or control (heterozygote or wild type; n = 4) embryos. (C) Flow cytometry of embryonic thymocytes after 2 d of culture in Iscove’s medium supplemented with 8% FCS. 7-AAD and annexin V staining of E16 old homozygote 
Ptenfloxflox/Lck-Cre (n = 4) or control (heterozygote; white bars, n = 3) embryos as determined by flow cytometry.

Firmly expressed in the thymus of wild-type and heterozygous Pten⁄⁄ mice, but was absent in the thymus of Ptenfloxflox/Lck-Cre mice (Fig. 1 B).

Conversion of PtdIns(4,5)P2 to PtdIns(3,4,5)P3 by PI-3K creates binding sites for PH domain proteins Akt/PKB, Tec, and Itk, which may result in activation of these enzymes. Because the absence of PTEN causes sustained PI-3K signaling, it is possible that one or more of these PH domain enzymes are constitutively activated in the thymus of Ptenfloxflox/Lck-Cre mice. Therefore, we compared the phosphorylation of Akt/PKB, Itk, and Tec in thymocytes of Ptenfloxflox/Lck-Cre and in Ptenfloxflox/+ Lck-Cre or wild-type mice. As a control, we included the human PTEN-deficient T cell line Jurkat (27), incubated or not with anti-CD3 antibody. Fig. 1 C demonstrates that the thymus of both 5-wk-old Ptenfloxflox/Lck-Cre mice that had no signs of tumors and tumor-bearing 14-wk-old Ptenfloxflox/Lck-Cre mice contained much higher levels of phosphorylated Akt/PKB than the thymus of heterozygous or wild-type control littermates. In contrast, almost no phosphorylated Itk (Fig. 1 D) or Tec (not depicted) could be detected in the thymus of 5-wk-old Ptenfloxflox/Lck-Cre mice. However, some phosphorylated Itk was observed in thymic tumor-bearing 14-wk-old mice. These data clearly indicate that the absence of PTEN leads to an increase of basal PtdIns(3,4,5)P3 levels in the thymus, resulting in an enhanced Akt/PKB phosphorylation.

Development of Lymphomas. To determine the impact of the Pten mutation, 20 Ptenfloxflox/Lck-Cre mice (10 males and 10 females) presenting a T cell–specific deletion were followed during their development. The first clinical signs of tumor formation were observed in some mice at 6–7 wk, and all the mice died within 17 wk (unpublished data).

The thymuses of Ptenfloxflox/Lck-Cre mice were analyzed before 6 wk of age. Ptenfloxflox/Lck-Cre mice analyzed at 1–6 wk did not show any signs of tumor formation. Importantly, thymus weight; thymocyte number; CD3, CD4, and CD8 phenotypes; and TCRβ diversity of thymocytes from Ptenfloxflox/Lck-Cre mice before 6 wk of age were completely comparable to those of Pten+/−/Lck-Cre mice (unpublished data), indicating that before the onset of lymphomas the PTEN deficiency does not lead to thymus hypercellularity.

Early T Cell Differentiation in Ptenfloxflox/Lck-Cre Mice. To investigate the possibility that PTEN deletion affects T cell development before the DP stage, we analyzed the DN compartment in thymocytes of Ptenfloxflox/Lck-Cre thymuses with antibodies against CD4, CD25 after exclusion of cells that express CD4 and CD8, TCRβ and NK (DX5) cells, granulocytes and plasmacytoid DCS (GR1), macrophages (MAC1), and B lymphocytes (B220). We frequently observed an increase in the percentage of CD4+CD25−DN4 thymocyte population in Ptenfloxflox/Lck-Cre mice compared to Pten+/−/Lck-Cre mice. Numbers indicate percentages of gated populations. (D) Percentages of iCTCRβ+ DN, ISP, and DP thymocytes of E16 old homozygote Ptenfloxflox/Lck-Cre (black bars, n = 4) or control (heterozygote; white bars, n = 3) embryos as determined by flow cytometry.
with heterozygous or wild-type mice, but these differences were not statistically significant (unpublished data).

Thus, in the steady state thymus, no significant differences between Pten<sup>flox/flox</sup>Lck-Cre and heterozygous and wild-type animals were observed with regard to the thymus size and distribution of various CD4 and CD8, DN, DP, and single positive (SP) populations. This was unexpected in view of the role of PtdIns(3,4,5)<sub>π</sub> and single positive (SP) populations. Therefore, we considered the possibility that Pten deletion affects the formation of the DP compartments during ontogeny. An analysis of DP thymocytes in Pten<sup>flox/flox</sup>Lck-Cre thymuses at day E16, when the thymus is being generated, revealed that the thymuses of E16 Pten<sup>flox/+ Lck-Cre</sup> embryos have 1.8–6-fold more DP cells (mean calculated from three Pten<sup>flox/flox</sup>Lck-Cre and four Pten<sup>flox/+ Lck-Cre</sup> embryos) as compared with thymuses of heterozygous or wild-type embryos (Fig. 2, A and B), suggesting that the absence of PTEN results in accelerated generation of DP thymocytes during ontogeny. To obtain information about the underlying mechanism, we tested the viability of the fetal thymocytes after 2 d of culture in Iscove’s medium plus 8% FCS. After the incubation, the cells were stained with annexin V and 7-AAD and analyzed by FACS (Fig. 2 C). The average number of viable cells in the cultured Pten<sup>/−</sup> thymocytes (48.3 ± 8.5, n = 4) was significantly higher than in the cultured control Pten<sup>/+</sup> thymocytes (26.4 ± 4.5, n = 3). These data suggest that the absence of PTEN confers a survival advantage to embryonic thymocytes. Loss of PTEN induces survival and proliferation of TCRβ<sup>−</sup> DP cells in mice compromised in pre-TCR signaling (see Expansion of icTCRβ<sup>−</sup> DP Thy-mocytes in Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>−</sup>/− Mice). These TCRβ<sup>−</sup> cells are in wild-type thymus eliminated after β-selection, but may survive and proliferate in embryonic Pten<sup>/−</sup> thymus. To address the question of whether the increase in DP cell numbers was due to a selective expansion of DP cytoplasmic (ic)TCRβ<sup>−</sup> cells, we analyzed the expression of icTCRβ in the embryonic Pten<sup>/−</sup> and Pten<sup>/+</sup> immature single positive (ISP) and DP cells (Fig. 2 D). The percentages of icTCRβ<sup>−</sup> cells were slightly higher in the Pten<sup>/−</sup> (58 ± 2.2) than in the Pten<sup>/+</sup> (78 ± 0.7) ISP compartment. However, the percentages of icTCRβ<sup>−</sup> cells in the DP compartment were similar in both groups of embryos (84 ± 1.8 of Pten<sup>/−</sup> and 88.5 ± 1.3 of Pten<sup>/+</sup> DP cells; Fig. 2 D). Thus, although we observed some increased survival of icTCRβ<sup>−</sup> cells in the ISP compartment of Pten<sup>flox/flox</sup>Lck-Cre embryos, the increase in DP cells ob-

![Figure 3](image-url)

**Figure 3.** The absence of PTEN in thymocytes can rescue the β-selection defect in CD3γ<sup>−</sup> mice. (A) Thymic cellularity of 1- or 3-wk-old Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>−</sup> mice (n = 6) compared with Pten<sup>flox/flox</sup>Lck-Cre or Pten<sup>/−</sup> (n = 4) and CD3γ<sup>−</sup> (n = 8) mice. (B) Flow cytometry of thymocytes. CD4CD8<sup>−</sup> and CD44, CD25 staining of 3-wk-old CD3γ<sup>−</sup> (n = 3), or Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>−</sup> mice (n = 4) mice. Numbers in quadrants indicate percentages of each population. Note that CD25 and CD44 were analyzed after gating on CD4<sup>+</sup>CD8<sup>+</sup> thymocytes. The gates were set to include 99% of the control, isotype-stained cells of each sample in the negative quadrant. (C) Flow cytometry of thymocytes. CD4CD8 staining of 3-wk-old control (heterozygote; n = 3), Pten<sup>flox/flox</sup>Lck-Cre (n = 4), CD3γ<sup>−</sup> (n = 4), or Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>−</sup> (n = 4) mice. Numbers in quadrants indicate percentages of each positive population.
served in Pten<sup>flox/flox</sup> embryonic thymus is not caused by a selective expansion of TCRβ<sup>+</sup> DP cells.

**Loss of Pten Rescues Thymic Cellularity in CD3γ<sup>-</sup>/Mice.** One explanation for the high numbers of DP thymocytes observed in E16 Pten<sup>flox/flox</sup>Lck-Cre mouse embryos was that elevated PtdIns(3,4,5)P<sub>3</sub> levels stimulate differentiation, cell survival, and/or proliferation around the β-selection checkpoint. To test this, we crossed Pten<sup>flox/flox</sup>Lck-Cre mice with CD3γ<sup>-</sup> mice that have a small thymus due to a poor capacity of inducing β-selection (20). Strikingly, the number of thymocytes in mice deficient for both Pten and CD3γ were increased 3–6-fold at 1 wk of age (15–30 × 10<sup>6</sup> cells in the double deficient mice vs. 5 × 10<sup>6</sup> in CD3γ<sup>-/-</sup>) to >20-fold (100–150 × 10<sup>6</sup> cells in Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> mice) at 3 wk of age compared with CD3γ<sup>-/-</sup> mice (Fig. 3 A). Analysis of CD4/CD8 distribution in these mice revealed that the percentages of DP cells in the thymus of mice deficient for both Pten and CD3γ were increased 40-fold compared with those in CD3γ<sup>-/-</sup> and similar to those of wild-type mice (Fig. 3 B, top). In addition, the percentages of DN4 thymocytes were strongly increased in the Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> mice (58% compared with 2% in CD3γ<sup>-/-</sup> mice; Fig. 3 B, bottom). These data indicate that the loss of Pten completely neutralized the effect of CD3γ deficiency on the generation of DN4 cells and the DP thymocytes.

It has been documented that CD25 is down-regulated in mature T cells, thus the loss of PTEN may affect CD25 expression as well. We observed that CD25 was absent on DP thymocytes in Pten<sup>flox/flox</sup>Lck-Cre mice with CD3γ<sup>-/-</sup> mice. It has been previously reported that CD25 is absent on mature T cells and that CD25<sup>-/-</sup> mice have a profoundly diminished thymus. Based on this, we believe that the difference in CD25 expression between wild-type and Pten<sup>flox/flox</sup>Lck-Cre mice is due to the absence of PTEN.

**Expansion of icTCRβ**<sup>-</sup> **DP Thymocytes in Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> Mice.** We reasoned that the presence of Pten in CD3γ<sup>-/-</sup> mice was due to the absence of PTEN in the pre-TCR complex. Activation of T cells through the mature TCR results in activation of PI-3K, PKB (9, 29, 30). Although there is no evidence yet that triggering of the pre-TCR results in activation of PI-3K and Akt/PKB, it was possible that increased basal levels of PtdIns(3,4,5)P<sub>3</sub> could amplify the suboptimal pre-TCR signal in CD3γ<sup>-/-</sup> mice. The second explanation was that Pten deficiency led to survival and proliferation not only of those cells that undergo β-selection but also of those that are normally eliminated during β-selection. The first explanation predicted that the majority of the DP cells in the thymus of Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> mice express TCRβ protein. If the second explanation was correct, we expected that many of the DP cells lack TCRβ protein. Assuming that 2/3 of the rearrangements at one TCR and 46% of the DP cells were TCR<sup>+</sup> at 3 wk of age compared with 61–88% of the DP cells in these mice were subjected to β-selection. This could have been due to signaling through the incomplete CD3δ, e-containing pre-TCR complex (31), which may induce selective survival but no proliferation of cells expressing a functional TCRβ/pTα dimer. In contrast, in Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> mice only 23–46% of the DP cells were TCRβ<sup>+</sup> (Fig. 4 and Table I). The increase in icTCRβ<sup>-</sup> cells was not due to a preferential outgrowth of TCRγδ<sup>+</sup> cells because no icTCRδ<sup>+</sup> DP cells were observed in the thymus of Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> mice (unpublished data). These data suggest that in the absence of PTEN, thymocytes lacking productive TCRβ rearrangements are able to survive and to expand over time in the DP stage. The data presented in Fig. 4 suggests that the presence of PTEN results in a selective outgrowth of TCRβ<sup>-</sup> DP thymocytes. However, inspection of the absolute numbers of icTCRβ<sup>+</sup> and icTCRβ<sup>-</sup> DP thymocytes in Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> and CD3γ<sup>-/-</sup> mice indicated that the numbers of icTCRβ<sup>+</sup> DP thymocytes were considerably increased in the absence of PTEN (Table I). At 1 wk of age, the numbers of icTCRβ<sup>+</sup> DP cells in the thymuses of both mice were similar; however, at 3–4 wk of age, the numbers of icTCRβ<sup>+</sup> DP thymocytes in the Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> mice were much higher (13 × 10<sup>6</sup> and 30 × 10<sup>6</sup>, respectively) than in the CD3γ<sup>-/-</sup> mice (0.64 × 10<sup>6</sup> and 0.08 × 10<sup>6</sup>, respectively). Thus, although the proportion of the icTCRβ<sup>-</sup> cells in the DP compartment decreased as a result of Pten deletion in CD3γ<sup>-/-</sup> background, the absolute numbers of these cells increased as compared with the numbers of DP thymocytes in CD3γ<sup>-/-</sup> mice with normal PTEN levels.

**Loss of PTEN Rescues Thymic Cellularity in γ<sup>-/-</sup> Mice.** How did the icTCRβ<sup>-</sup> cells survive in Pten<sup>flox/flox</sup>Lck-Cre × CD3γ<sup>-/-</sup> mice? One possibility was that Pten deficiency mimicked the IL-7R signal, which is normally absent in wild-type nonselected DN3 cells. It has been established that IL-7 activates PI-3K in thymocytes (32).
is analyzed on CD4 complex. To test this idea, we analyzed the thymus of trol (wild type; cytometry of thymocytes. Intracellular TCR (Fig. 5 B) with normal percentages of various TCR /H9253/H9254 eage thymocyte subsets (Fig. 5 A). Interestingly, TCR Ins(3,4,5) 1 might have been possible that the absence of PTEN, which catalyzes the reverse conversion of PtdIns(3,4,5) P3 from three different litters at the age of 4–5 wk. The thy- muses of these three mice contained 50 × 10^6 (4 wk), 95 × 10^6 (5 wk), and 123 × 10^6 (5 wk) cells, respectively. The thymus phenotypes of these mice were identical. Fig. 6 A shows that loss of PTEN compensated for the loss of both γc and the pre-TCR with regard to the numbers of thymocytes. Most of the thymocytes in the Pten^floxfloxLck-Cre × γc^-/- × Rag2^-/- mice were DP (70%), but also some CD4+ and CD8+ SP cells could be observed (Fig. 6 A). As expected, the DP thymocytes expressed iCDS, but did not express cell surface CD3e nor iTCRβ (Fig. 6 A and not depicted), confirming the absence of a pre-}

### Table I. Thymus Cell Counts, Percentages, and Absolute Cell Numbers of CD4^+CD8^+ iTCRβ^+ or CD4^+CD8^+ iTCRβ^- Cells in Wild Type, Pten^floxfloxLck-Cre, CD3γ^-/-, and Pten^floxfloxLck-Cre × CD3γ^-/- Mice

| Age (wk) | Genotype | n | Thymus cellularity | CD4^+CD8^+ total | CD4^+CD8^+ iTCRβ^- | CD4^+CD8^+ iTCRβ^+ | CD4^+CD8^+ iTCRβ^- | CD4^+CD8^+ iTCRβ^+ |
|----------|----------|---|--------------------|------------------|------------------|------------------|------------------|------------------|
|          |          |   | no. × 10^-6 | % | % | no. × 10^-6 | no. × 10^-6 |
| 1        | wild type | ND | ND | ND | ND | ND | ND |
|          | Pten^-/-  | ND | ND | ND | ND | ND | ND |
|          | CD3γ^-/-  | 9  | 4.4 ± 1.4 | 12 ± 4.4 | 88 ± 2.7 | 0.48 ± 0.26 | 0.07 ± 0.04 |
|          | Pten^-/- × CD3γ^-/- | 1  | 3.4 | 26 | 46 | 0.40 | 0.47 |
| 2        | wild type | 7  | 96 ± 20 | 81 ± 5.6 | 99 ± 0.1 | 80 ± 15 | 0.3 ± 0.1 |
|          | Pten^-/-  | 4  | 108 ± 10 | 81 ± 6.8 | 99 ± 0.1 | 88 ± 13 | 0.3 ± 0.1 |
|          | CD3γ^-/-  | 7  | 12 ± 2.4 | 87 ± 7.7 | 58 ± 5.1 | 0.64 ± 0.56 | 0.44 ± 0.41 |
|          | Pten^-/- × CD3γ^-/- | 3  | 56 ± 23 | 83 ± 1.5 | 27 ± 9.1 | 13 ± 7.1 | 34 ± 16 |
| 4        | wild type | 3  | 144 ± 32 | 81 ± 0.9 | 99 ± 0.9 | 114 ± 23 | 2.0 ± 1.2 |
|          | Pten^-/-  | 2  | 162 ± 29 | 78 ± 3.3 | 99 ± 0.4 | 124 ± 27 | 1.6 ± 0.8 |
|          | CD3γ^-/-  | 4  | 5.4 ± 0.3 | 2.0 ± 0.7 | 70 ± 7.0 | 0.08 ± 0.03 | 0.03 ± 0.003 |
|          | Pten^-/- × CD3γ^-/- | 3  | 180 ± 4.0 | 72 ± 5.0 | 23 ± 3.0 | 30 ± 5.3 | 100 ± 0.9 |

Moreover, PI-3K can associate with the IL-7Rα after engagement with IL-7 (33) and a dominant negative form of p85 inhibited T cell development (8), strongly suggesting that accumulation of PtdIns(3,4,5)P3 is critical for IL-7–mediated survival and proliferation of early T cell precursors. It might have been possible that the absence of PTEN, which catalyzes the reverse conversion of PtdIns(3,4,5)P3 into PtdIns(3,4,5)P2 leading to elevated basic levels of PtdIns(3,4,5)P3, would compensate for the loss of the IL-7R complex. To test this idea, we analyzed the thymus of γc^-/- mice crossed with Pten^floxfloxLck-Cre mice. The absence of PTEN in γc^-/- background rescued thymic cellularity (Fig. 5 B) with normal percentages of various TCRαβ lineage thymocyte subsets (Fig. 5 A). Interestingly, TCRγδ cells were not rescued by the absence of PTEN in γc^-/- mice (Fig. 5 A). IL–7 (34) and its receptor (35–37) are re-
PTEN deficiency substitutes for IL-7 and pre-TCR signals. Because the up-regulation of CD2 and CD5 and the down-regulation of CD25 are considered to be hallmarks of pre-TCR expression, we also analyzed the expression of CD2, CD5, and CD25. Fig. 6B shows that CD2 and CD5 were only very slightly up-regulated and much less than in wild-type DP thymocytes. CD25 was not expressed on DP cells of Pten<sup>flox/flox</sup> <sup>Lck-Cre</sup> mice. The DP cells of these mice expressed almost no CD69 (unpublished data), which was expected because the activation marker CD69 is only up-regulated as consequence of TCR-mediated positive selection. Despite the presence of small numbers of SP cells in the thymus of Pten<sup>flox/flox</sup> <sup>Lck-Cre</sup> × γ<sup>c</sup>-/- × Rag2<sup>2/-</sup> mice, no CD4<sup>+</sup> or CD8<sup>+</sup> cells could be found in the spleen of these mice (Fig. 6C). We conclude that the loss of PTEN resulted in proliferation of thymocytes and induction of CD4 and CD8 in the absence of IL-7R and pre-TCR signaling.

Discussion
In this work, we demonstrate a critical role of PTEN in regulation of survival and growth of developing T cells in the thymus. We have analyzed mice with a T cell lineage-specific PTEN deletion. In agreement with the observations of Suzuki et al. (17), we observed that all Pten<sup>flox/flox</sup> Lck-Cre mice developed T cell lymphomas. A comparison of wild-type and Pten<sup>flox/flox</sup> Lck-Cre mice before the onset
of lymphomagenesis revealed no gross differences in thymic cellularity and distribution of various DN, DP, and SP populations. Using an independently made mouse strain with a T cell–specific loss of PTEN, Suzuki et al. also noted little effects on the phenotypes of thymocytes, but these investigators observed a modest hypercellularity of the thymus before the onset of lymphomagenesis (38). Our data indicate that loss of PTEN does not affect thymic cellularity and the distribution of CD4 and CD8 under steady state conditions. Thus, PtdIns(3,4,5)P3 levels produced in wild-type mice are not rate limiting for optimal proliferation of developing T cells. However, we observed a higher number of DP cells in thymuses of Pten<sup>lox/lox</sup>Lck-Cre E16 embryos compared with heterozygous or wild-type E16 embryos, suggesting that PTEN deficiency conferred a proliferative advantage to early pre–T cells before and/or after the β-selection checkpoint during ontogeny. To examine this, we introduced the Pten deletion in mice with deficiencies in IL-7R, pre-TCR signaling, or both. The size of the thymus was strongly increased in the absence of PTEN in either context, indicating the importance of sustained PtdIns(3,4,5)P3 levels for expansion of thymocytes at all stages of differentiation. The observation that the absence of PTEN compensated for the effect of γδ deletion on thymic cellularity is consistent with the notion that PI-3K is pivotal for the IL-7–induced proliferation of pre–T cells (8). However, TCRγδ cells were not rescued. Assuming that the Lck-Cre transgene is also expressed in TCRγδ cells, our findings indicate that Pten deletion did not recapitulate all effects of the IL-7R. This was expected because the absence of TCRγδ cells in γδ<sup>−/−</sup> mice is the result of inefficient rearrangements at the TCRγ locus (34–37), which is mediated in wild-type mice through activation of STAT5 by IL-7 (39, 40). However, we cannot formally exclude that in our mice the Lck-Cre transgene was not expressed in TCRγδ cells.

The deficiency of PTEN in CD3γ<sup>−/−</sup> mice and mice with a RAG2 deficiency eventually resulted in a numerical reconstitution of the thymus and high percentages of DP cells, indicating that the appearance of CD4 and CD8 is a consequence of increased PtdIns(3,4,5)P3 levels resulting from the loss of PTEN. Strikingly, we observed that the proportion of TCRβ<sup>−</sup> DP cells in the thymus of Pten<sup>lox/lox</sup>Lck-Cre × CD3γ<sup>−/−</sup> mice is much higher than in CD3γ<sup>−/−</sup> mice. These TCRβ<sup>−</sup> DP cells were also negative for iTCRδ, but expressed iCD3e. Moreover, loss of PTEN also rescued the thymic cellularity in mice deficient for RAG2 and γδ, which do not express a pre-TCR at all. These findings indicate that the absence of PTEN results in

---

**Figure 6.** Loss of PTEN compensates the thymic defect in γδ<sup>−/−</sup> × Rag2<sup>−/−</sup> mice. (A) Flow cytometric analysis of expression of CD4CD8, iCD3e, and iTCRβ in thymocytes of 4–5 wk-old control (wild type; n = 11), γδ<sup>−/−</sup> × Rag2<sup>−/−</sup> (n = 1), and Pten<sup>lox/lox</sup>Lck-Cre × γδ<sup>−/−</sup> × Rag2<sup>−/−</sup> (n = 3) mice. Numbers in quadrants indicate percentages of each population. The total numbers of thymocytes are indicated on top of the CD4/CD8 dotplots. The gates were set to include 99% of the control, isotype-stained cells of each sample in the negative quadrant. (B) Expression of CD2, CD5 and CD25 in CD4<sup>+</sup>CD8<sup>+</sup> cells of 4–5 wk-old control (wild type; n = 11) and Pten<sup>lox/lox</sup>Lck-Cre × γδ<sup>−/−</sup> × Rag2<sup>−/−</sup> (n = 3) mice. The cells were stained and expression of CD2, CD5 and CD25 were analyzed on CD4<sup>−</sup>CD8<sup>−</sup> DP thymocytes. (C) Expression of CD4 and CD8 on splenic cells of 4–5 wk-old control (wild type; n = 11) and Pten<sup>lox/lox</sup>Lck-Cre × γδ<sup>−/−</sup> × Rag2<sup>−/−</sup> (n = 3) mice. Numbers in quadrants indicate percentages of each population. The gates were set to include 99% of the control, isotype-stained, cells of each sample in the negative quadrant.
survival and expansion of early TCRαβ lineage cells that are normally eliminated during β-selection. It is important to note that, whereas the proportion of iTCRβ+ cells was decreased, the absolute numbers of these cells were increased in \textit{Pten\textsuperscript{flox/flox}Lck-Cre} × \textit{CD3γ−/−} mice compared with \textit{CD3γ−/−} mice (Table I), indicating that the absence of PTEN in the thymus results in survival and expansion of cells both with productive (iTCRβ+) and unproductive TCRβ (iTCRβ−) rearrangements. We have observed that the absence of PTEN reconstituted thymic cellularity in γ−/− mice and, thus, compensated for the absence of the IL-7–mediated survival and proliferation signals.

These data may explain why PTEN deficiency leads to survival of iTCRβ− cells in the context of suboptimal pre-TCR signaling and suggests a physiological role of PTEN in β-selection. Approximately one third of TCRβ rearrangements at the first allele are successfully producing a full-length TCRβ protein (2). Assuming that one third of the cells that fail to successfully complete a TCRβ rearrangement at one allele complete a productive rearrangement at the second allele, five out of nine cells eventually produce a TCRβ protein, the rest being eliminated (2). It seems reasonable to assume that these cells die because of an absence of a survival signal. This implies that IL-7R signaling needs to be shut off in cells that failed to pass the β-selection. The currently accepted model holds that cells expressing a functional TCRβ–pTα complex are rescued from death by pre-TCR signaling and proliferate, whereas the nonselected cells, which do not receive a survival signal neither from the IL-7R nor from the pre-TCR will die.

A problem with this model is that, although the IL-7R is down-regulated in iTCRβ− DN4 cells compared with iTCRβ+ DN4 cells, there is still some IL-7R expressed on iTCRβ− DN4 cells (41), raising the question how IL-7R signaling is turned off in pre–T cells poised for elimination. We propose that PTEN shuts off a remaining IL-7–mediated survival signal and ensures that the pre–T cells that failed to complete productive rearrangements at the TCRβ locus cannot receive a survival signal and, thus, die by neglect. The observation of a dramatic expansion of DP cells in \textit{Pten\textsuperscript{flox/flox}Lck-Cre} × \textit{γc−/−} × \textit{Rag2−/−} mice is consistent with this model. However, it should be noted that we did not observe increased numbers of TCRβ− cells in DP \textit{Pten\textsuperscript{flox/flox}Lck-Cre} thymocytes compared with wild-type cells.

To account for this observation in the context of our hypothesis, we propose that the signal induced by an intact pre-TCR results in a much higher rate of proliferation than induced by the mere absence of PTEN. Because of the difference in proliferation rate, the DP cells expressing an intact pre-TCR in \textit{Pten\textsuperscript{flox/flox}Lck-Cre} mice preferentially filled the DP “niche,” whereas in \textit{Pten\textsuperscript{flox/flox}Lck-Cre} × \textit{CD3γ−/−} mice, the TCRβ− cells did not have a proliferative advantage and hence the DP niche was filled with both TCRβ+ and TCRβ− cells. This notion is supported by the observation that the CD3γ deficiency was only fully compensated by the absence of PTEN 3 wk after birth (Fig. 4). Thus, before the first 2 wk after birth, PTEN-deficient thymocytes that undergo normal pre-TCR signaling had expanded much more than the PTEN CD3γ doubly deficient thymocytes in which pre-TCR signaling is compromised.

Our data indicate that the absence of PTEN sufficiently elevates basal levels of PtdIns(3,4,5)P3 to mediate survival and proliferation of thymocytes before and after the β-selection point in the absence of external growth stimuli. Any signaling molecule that has a PI domain that preferentially binds to PtdIns(3,4,5)P3 can be involved in expansion of thymocytes observed in the \textit{Pten}−/− mice (42). PDK-1 is a “master” kinase that phosphorylates residues in the activation loops of AGC superfamily serine/threonine kinases, including the PI-3K–controlled serine kinases Akt/PKB, which are corecruited to PtdIns(3,4,5)P3, and S6 kinase 1. Indeed, Akt/PKB is phosphorylated in the thymus of \textit{Pten\textsuperscript{flox/flox}Lck-Cre} mice, indicating that PDK-1 is active. At least 13 substrates of Akt/PKB have been identified so far and can be separated into two main subsets: regulators of survival/apoptosis and cell cycle regulators (for review see reference 43), giving to Akt/PKB an important role in the control of the survival/proliferation of different cell types. Expression of a transgene encoding a constitutive active Akt/PKB (gagPKB) has been shown to improve survival of thymocytes and mature T lymphocytes (9). However, introduction of a transgene encoding another constitutively membrane-targeted Akt/PKB (myristoylated Akt/PKB) in γc−/− mice or in pre–TCR−/− mice failed to reconstitute thymic cellularity in these animals (Di Santo, J., personal communication). Unless one assumes that myristoylated Akt/PKB, because of its forced membrane targeting, does not completely mimic the natural PtdIns(3,4,5)P3-recruited Akt/PKB in a thymic context, these data suggest that activated Akt/PKB by itself is not responsible for the generation of a full size thymus in \textit{Pten\textsuperscript{flox/flox}Lck-Cre} × γc−/− mice. Given the observations that the myristoylated Akt/PKB transgene failed to reconstitute thymic cellularity in γc−/− mice or in Rag2−/− mice, it is unlikely that known targets of Akt/PKB as Bad and Caspase 9 are involved in the effect caused by the loss of PTEN. The antiapoptotic molecule Bcl-2, believed to be downstream of the IL-7 receptor (44, 45) and possibly induced in a PI-3K–dependent way, is likely not a critical element as transgenic overexpression of Bcl-2 is unable to rescue the CD3γ (46), RAG (44), or γc deficiencies (7, 21). We could not detect phosphorylation of another PtdIns(3,4,5)P3-regulated kinase, Itk, in PTEN−/− thymocytes before the onset of lymphomagenesis, arguing against a role of this enzyme. We did observe some phosphorylated Itk in the thymus of 14-wk-old thymic tumor-bearing mice, but the mechanism remains to be established.

Our data suggest that PtdIns(3,4,5)P3-dependent molecules other than Akt/PKB or Itk are involved in the growth-promoting effects of thymocytes. Possible candidates
are the small GTPases Rac and Rho, which are influenced by PI-3K (47) and have been shown to affect growth of early T cell precursors (4, 48). Another possible mediator is the mammalian target of rapamycin (mTOR) because rapamycin reduces the size of the thymus in the mouse (49) and inhibits transition of DN to DP cells in the rodent thymus (50). Interestingly rapamycin interferes with GM-CSF signaling in DCs partly through down-regulation of the antipapoptotic molecule Mcl-1, indicating that in these cells, mTOR mediates expression of Mcl-1 (51). Recent data strongly suggest that Mcl-1 is involved in IL-7–mediated survival (52), but it is unclear whether Mcl-1 is involved in pre-TCR–mediated cell survival. Whether or not Mcl-1 levels are affected by the loss of PTEN is not yet known. Due to the complexity of the network of downstream signaling pathways that are connected to PI-3K and PTEN-dependent PtdIns(3,4,5)P3, determination of the exact downstream participants in the pathway that controls proliferation of T lineage cells during development will not be straightforward.

Given our observations and those of Hinton et al. indicating that PDK-1 deficiency strongly compromises proliferation and differentiation at the DN to DP transition (42), PDK-1 and PTEN may be considered to form a switch that functions as a major regulator of survival and proliferation of developing thymocytes. The absence of PTEN induces proliferation and is dominant over the apoptosis-inducing signals, which may be the reason why the PTEN deficiency leads to thymic tumors with a rapid onset.

We thank Drs. A. Kruisbeek and M. Haks for kindly providing the CD3γ−/− mice. We thank Drs. A. Berns, B. Blom, J. DiSanto, and D. Cantrell for critically reading the manuscript. We would like to thank Dr. L.J. Berg for providing reagents.

This work was supported by an EEC fellowship contract no. HPMF-CT-1999-00057 (to M. Naspetti); by grants from the Netherlands Foundation for Cancer Research, no. NKI 2000–2279, and the Dutch National Research Organization (NWO), no. 901–08–093 (to H. Spits); and a grant “Equipe labellisée 2001” from the Ligue Nationale Contre le Cancer (to F. Garcon and J. Nunes).

The authors have no conflicting financial interests.

Submitted: 16 March 2004
Accepted: 17 August 2004

References

1. Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. ImmunoL Today. 14:547–553.
2. Mallick, C.A., E.C. Dudley, J.L. Viney, M.J. Owen, and A.C. Hayday. 1993. Rearrangement and diversity of T cell receptor beta chain genes in thymocytes: a critical role for the beta chain in development. Cell. 73:513–519.
3. von Boehmer, H., I. Aifantis, J. Feinberg, O. Lechner, C. Saint-Ruf, U. Walter, J. Buer, and O. Azogui. 1999. Pleiotropic changes controlled by the pre-T-cell receptor. Curr. Opin. Immunol. 11:135–142.
4. Gomez, M., V. Tybulewicz, and D.A. Cantrell. 2000. Control of pre-T cell proliferation and differentiation by the GTPase Rac-1. Nat. Immunol. 1:348–352.
5. Kim, K., C.K. Lee, T.J. Sayers, K. Muegge, and S.K. Durham. 1998. The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways. J. Immunol. 160:5735–5741.
6. Offner, F., and J. Plum. 1998. The role of interleukin-7 in early T-cell development. Leuk. Lymphoma. 30:87–99.
7. Rodewald, H.R., C. Waskow, and C. Haller. 2001. Essential requirement for c-kit and common γ chain in thymocyte development cannot be overruled by enforced expression of Bcl-2. J. Exp. Med. 193:1431–1437.
8. Pallard, C., A.P. Stegmann, T. van Kleffens, F. Smart, A. Venkitaraman, and H. Spits. 1999. Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7–mediated development of human thymocyte precursors. Immunity. 10:525–535.
9. Jones, R.G., M. Parsons, M. Bonnard, V.S. Chan, W.C. Yeh, J.R. Woodgett, and P.S. Ohashi. 2000. Protein kinase B regulates T lymphocyte survival, nuclear factor κB activation, and Bcl-X(L) levels in vivo. J. Exp. Med. 191:1721–1734.
10. Myers, M.P., I. Pass, I.H. Barry, J. Van der Kaay, J.P. Stolzov, B.A. Hemmings, M.H. Wigler, C.P. Downes, and N.K. Tonks. 1998. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. Proc. Natl. Acad. Sci. USA. 95:13513–13518.
11. Cantley, L.C., and B.G. Neel. 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/akt pathway. Proc. Natl. Acad. Sci. USA. 96:4240–4245.
12. Di Cristofano, A., and P.P. Pandolfo. 2000. The multiple roles of PTEN in tumor suppression. Cell. 100:387–390.
13. Seminario, M.C., and R.L. Wange. 2003. Lipid phosphatases in the regulation of T cell activation: living up to their PTEN-tial. Immunol. Rev. 192:80–97.
14. Stambolic, V., A. Suzuki, J.L. de la Pompa, G.M. Brothers, C. Mirtsos, T. Sasaki, J. Ruland, J.M. Penninger, D.P. Sidировski, and T.W. Mak. 1998. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell. 95:29–39.
15. Di Cristofano, A., B. Pesce, C. Cordon-Cardo, and P.P. Pandolfo. 1998. Pten is essential for embryonic development and tumour suppression. Nat. Genet. 19:348–355.
16. Di Cristofano, A., P. Kotsi, Y.F. Peng, C. Cordon-Cardo, K.B. Elkon, and P.P. Pandolfo. 1999. Impaired Fas response and autoimmune in Pten+/- mice. Science. 285:2122–2125.
17. Suzuki, A., J.L. de la Pompa, V. Stambolic, A.J. Elia, T. Sasaki, I. del Barco Barrantes, A. Ho, A. Wakeham, A. Itie, W. Khoo, M. Fukumoto, and T.W. Mak. 1998. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr. Biol. 8:1169–1178.
18. Marino, S., P. Krimpenfort, C. Leung, H.A. Van Der Korput, J. Trapman, I. Camenisch, A. Berns, and S. Brandner. 2002. PTEN is essential for cell migration but not for fate determination and tumourigenesis in the cerebellum. Development. 129:3513–3522.
19. Gu, H., J.D. Marsh, P.C. Orban, H. Mossmann, and K. Rajewsky. 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science. 265:103–106.
20. Haks, M.C., P. Krimpenfort, J. Borst, and A.M. Kruisbeek. 1998. The CD3γ chain is essential for development of both the TCRαβ and TCRγδ lineages. EMBO J. 17:1871–1882.
21. Blom, B., H. Spits, and P. Krimpenfort. 1997. The role of the common gamma chain of the IL-2, IL-4, IL-7 and IL-15 receptors in development of lymphocytes: constitutive expression of Bcl-2 does not rescue the developmental defects in gamma common-deficient mice. *In Cytokines and Growth Factors in Blood Transfusion*. C.T. Smit Sibinga, P.C. Das, and B. Löwenberg, editors. Kluwer Academic Publishers, London. 3–12.

22. Kirberg, J., A. Berns, and H. von Boehmer. 1997. Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. *J. Exp. Med.* 186:1269–1275.

23. Horton, R.M., P.I. Karachunski, and B.M. Conti-Fine. 1995. PCR screening of transgenic RAG-2 “knockout” immunodeficient mice. *Biotechniques.* 19:690–691.

24. Nunes, J., S. Klasen, M. Raguenneau, C. Pavon, D. Couze, C. Mawas, M. Bagnasco, and D. Olice. 1993. CD28 mAbs with distinct binding properties differ in their ability to induce T cell activation: analysis of early and late activation events. *Int. Immunol.* 5:311–315.

25. Yang, W.C., M. Ghiotto, R. Castellano, Y. Collette, N. Aphn, J.A. Nunes, and D. Olice. 2000. Role of Tec kinase in nuclear factor of activated T cells signaling. *Int. Immunol.* 12: 1547–1552.

26. Ali, I.U., L.M. Schriml, and M. Dean. 1999. Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity. *J. Natl. Cancer Inst.* 91:1922–1932.

27. Seminario, M.C., P. Precht, R.P. Wermo, M. Gorospe, and R.L. Wange. 2003. PTEN expression in PTEN-null leukemic T cell lines leads to reduced proliferation via slowed cell cycle progression. *Oncogene.* 22:8195–8204.

28. Ward, S.G., S.C. Ley, C. MacPhee, and D.A. Cantrell. 1992. Regulation of D-3 phosphoinositides during T cell activation via the T cell antigen receptor/CD3 complex and CD2 antigen. *Eur. J. Immunol.* 22:45–49.

29. Kane, L.P., V.S. Shapiro, D. Stokoe, and A. Weiss. 1999. Induction of NF-kappaB by the Akt/PKB kinase. *Curr. Biol.* 9:601–604.

30. Lafont, V., E. Astoul, A. Laurence, J. Liatard, and D. Cantrell. 2000. The T cell antigen receptor activates phosphatidylinositol 3-kinase-regulated serine kinases protein kinase B and ribosomal S6 kinase 1. *FEBS Lett.* 486:38–42.

31. Haks, M.C., T.A. Cordaro, J.H. van den Brakel, J.B. Haanen, E.F. de Vries, J. Borst, P. Krimpenfort, and A.M. Kruisbeek. 2001. A redundant role of the CD3 gamma chain of the interleukin-7 receptor-deficient mice. *J. Exp. Med.* 194:692–701.

32. Reif, K., C.D. Nobes, G. Thomas, A. Hall, and D.A. Cantrell. 1996. Phosphatidylinositol 3-kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. *Curr. Biol.* 6:1445–1455.

33. Venkitaraman, A.R., and R.J. Cowling. 1994. Interleukin-7 induces the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. *Eur. J. Immunol.* 24:2168–2175.

34. Laky, K., L. Lefrancois, F. von, U. Jeffry, R. Murray, and L. Puddington. 1998. The role of IL-7 in thymic and extrathymic development of TCR gamma delta cells. *J. Immunol.* 161:707–713.

35. Maki, K., S. Sunaga, and K. Ikuta. 1996. The V-J recombination of T cell receptor-gamma genes is blocked in interleukin-7 receptor-deficient mice. *J. Exp. Med.* 184:2423–2427.

36. Durum, S.K., S. Candeias, H. Nakajima, W.J. Leonard, A.M. Baird, L.J. Berg, and K. Muegge. 1998. Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility. *J. Exp. Med.* 188:2233–2241.

37. Muljo, S.A., and M.S. Schlissel. 2000. Pre-B and pre-T-cell receptors: conservation of strategies in regulating early lymphocyte development. *Immunol. Rev.* 175:80–93.

38. Suzuki, A., M.T. Yamaguchi, T. Ohteki, T. Sasaki, T. Kaush, Y. Kimura, R. Yoshida, B. Wakeham, T. Higuchi, M. Fukushima, et al. 2001. T cell-specific loss of Pten leads to defects in central and peripheral tolerance. *Immunity.* 14:523–534.

39. Ye, S.K., K. Maki, T. Kitamura, S. Sunaga, K. Akashi, J. Domen, I.L. Weissman, T. Honjo, and K. Ikuta. 1999. Induction of germline transcription in the TCR gamma locus by Stat5: implications for accessibility control by the IL-7 receptor. *Immunity.* 11:213–223.

40. Lee, H.C., S.K. Ye, T. Honjo, and K. Ikuta. 2001. Induction of germline transcription in the human tgamma locus by Stat5. *J. Immunol.* 167:320–326.

41. Trigueros, C., K. Hozumi, B. Silva-Santos, L. Bruno, A.C. Haydaj, M.J. Owen, and D.J. Pennington. 2003. Pre-TCR signaling regulates IL-7 receptor alpha expression promoting thymocyte survival at the transition from the double-negative to double-positive stage. *Eur. J. Immunol.* 33:1968–1977.

42. Hinton, H.J., D.R. Alessi, and D.A. Cantrell. 2004. The serine kinase phosphoinositide-dependent kinase 1 (PDK1) regulates T cell development. *Nat. Immunol.* 5:539–545.

43. Blume-Jensen, P., and T. Hunter. 2001. Oncogenic kinase signalling. *Nature.* 411:355–365.

44. Maraskovsky, E., L.A. O’Reilly, M. Corcoran, J.J. Peschon, and A. Strasser. 1997. Bcl-2 can rescue T lymphocyte development in interleukin-7 receptor-deficient mice but not in mutant rag-1−/− mice. *Cell.* 89:1011–1019.

45. Akashi, K., M. Kondo, F. von, U. Jeffry, R. Murray, and I.L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell.* 89:1033–1041.

46. Haks, M.C., P. Krimpenfort, J.H. van den Brakel, and A.M. Kruisbeek. 1999. Pre-TCR signaling and inactivation of p53 induces crucial cell survival pathways in pre-T cells. *Immunity.* 11:91–101.

47. Damiaseaux, J.G., L.J. Beijleveld, H.J. Schuurman, and P.J. van Breda Vriesman. 1996. Effect of in vivo rapamycin treatment on de novo T-cell development in relation to inducible CRTH2 expression. *Eur. J. Immunol.* 26:2168–2175.

48. Damiaseaux, J.G., L.J. Beijleveld, H.J. Schuurman, and P.J. van Breda Vriesman. 1996. Effect of in vivo rapamycin treatment on de novo T-cell development in relation to induction of autoimmune-like immunopathology in the rat. *Transplantation.* 62:994–1001.

49. Woltman, A.M., S.W. van der Kooij, P.J. Coffer, R. Oftringa, M.R. Daha, and C. van Kooten. 2003. Rapamycin specifically interferes with GM-CSF signaling in human dendritic cells, leading to apoptosis via increased p27kip1 expression. *Blood.* 101:1439–1455.

50. Opferman, J.T., A. Letai, C. Beard, M.D. Sorcinelli, C.C. Ong, and S.J. Korsmeyer. 2003. Development and maintenance of B and T lymphocytes requires ant apoptotic MCL-1. *Nature.* 426:671–676.