

Fit $\alpha\beta$ T-cell receptor suppresses leukemogenesis of Pten-deficient thymocytes

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ABSTRACT

Signaling through the $\alpha\beta$ T cell receptor (TCR) is a crucial determinant of T-cell fate and can induce two opposite outcomes during thymocyte development: cell death or survival and differentiation. To date, the role played by T-cell receptor in the oncogenic transformation of developing T cells remains unclear. Here we show that human primary T-cell acute lymphoblastic leukemias expressing an $\alpha\beta$ T cell receptor are frequently deficient for phosphatase and tensin homolog protein (PTEN), and fail to respond strongly to T-cell receptor activation. Using Pten-deficient T-cell acute lymphoblastic leukemia mouse models, we confirm that T-cell receptor signaling is involved in leukemogenesis. We show that abrogation of T-cell receptor expression accelerated tumor onset, while enforced expression of a fit transgenic T-cell receptor led to the development of T-cell receptor-negative lymphoma and delayed tumorigenesis. We further demonstrate that pre-tumoral Pten-deficient thymocytes harboring fit T-cell receptors undergo early clonal deletion, thus preventing their malignant transformation, while cells with unfit T-cell receptors that should normally be deleted during positive selection, pass selection and develop T-cell acute lymphoblastic leukemias. Altogether, our data show that fit T-cell receptor signaling suppresses tumor development mediated by Pten loss-of-function and point towards a role of Pten in positive selection.

Introduction

Thymopoiesis aims to create a repertoire of mature T cells equipped with a diverse array of functional $\alpha\beta$ or $\delta\gamma$ T-cell receptors (TCR) able to recognize the broadest possible range of foreign antigens. This large receptor diversity is mainly due to the V(D)J recombination process, in which few of a large pool of V, D and J gene segments are somatically rearranged with imprecise joining. The price to pay for this strategy of random generation of diversity is the creation of a high load of CD4⁺ CD8⁺ DP cortical thymocytes bearing no, or “unfit” receptors, i.e. displaying a too low or too high affinity for self peptide-major histocompatibility complex (p-MHC), and which will have to be eliminated through death by neglect (>90% thymocytes) and negative selection (*Online Supplementary Figure S1A*).¹ Only the small pool of thymocytes expressing TCR with intermediate affinity and/or avidity for p-MHC (denoted here as fit TCR) will be induced to further differentiate into mature CD4 or CD8 single-positive (SP) thymocytes, a transition known as positive selection. Therefore, TCR signaling can induce two opposite outcomes during thymocyte development: cell death or survival and differentiation.

Somatic rearrangement, proliferation and selection provide a propitious environment for major derailments of thymocyte ontogeny. T-cell acute lymphoblastic leukemias (T-ALL) are malignant proliferation of such T-cell progenitors abnormally arrested at various stages of their maturation process (*Online Supplementary Figure S1B*).^{2,4} They constitute a particularly heterogeneous group of diseases, resulting



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from a large array of genetic and epigenetic alterations in oncogenes and tumor suppressors.⁵⁻⁷ A wealth of information has emerged from recent pan-(epi)genomic analysis of T-ALLs, and this has allowed mapping of cell-intrinsic genetic defaults in these cells to be expanded. However, much less is known about the potential cell-extrinsic cues that may impact on the leukemia genesis process, including the role that the TCR might play in malignant transformation throughout thymocyte selection, survival and proliferation.⁸⁻¹⁰ In this study, we sought to address how TCR signaling can interfere or, on the contrary, can be integrated in T-ALL oncogenic networks.

Methods

Patients' samples

Diagnostic specimens (peripheral blood or bone marrow) collected from patients treated at the Timone Children's Hospital or Paoli Calmettes Institute (Marseille, France) or from Necker Hospital (Paris, France) were used to generate xenografts. Diagnosis and classification were defined by expression of specific

T-cell markers and negativity for B cells and myeloid markers. Healthy human thymus were obtained from Timone Children's Hospital. Samples were purified by Ficoll-Hypaque centrifugation. T-ALLs were included within FRALLE-2000 or GRAALL-2005 protocols, and informed consent for use of diagnostic specimens for future research was obtained from the patients or relatives in accordance with the Declaration of Helsinki. This study was approved by institutional review boards of all hospitals involved.

Mice

Mice were bred and housed in specific pathogen-free conditions in CIML animal facilities and were handled in accordance with French and European guidelines. Mice strains and oligonucleotides used for mice genotyping are listed in the *Online Supplementary Methods* and *Online Supplementary Table S1*. Xenotransplantation of primary human T-ALL samples in immunodeficient NSG mice was performed as previously described.¹¹

TCR-signaling ability assays

TCR-signaling ability assays were performed with 2×10^7 wild-

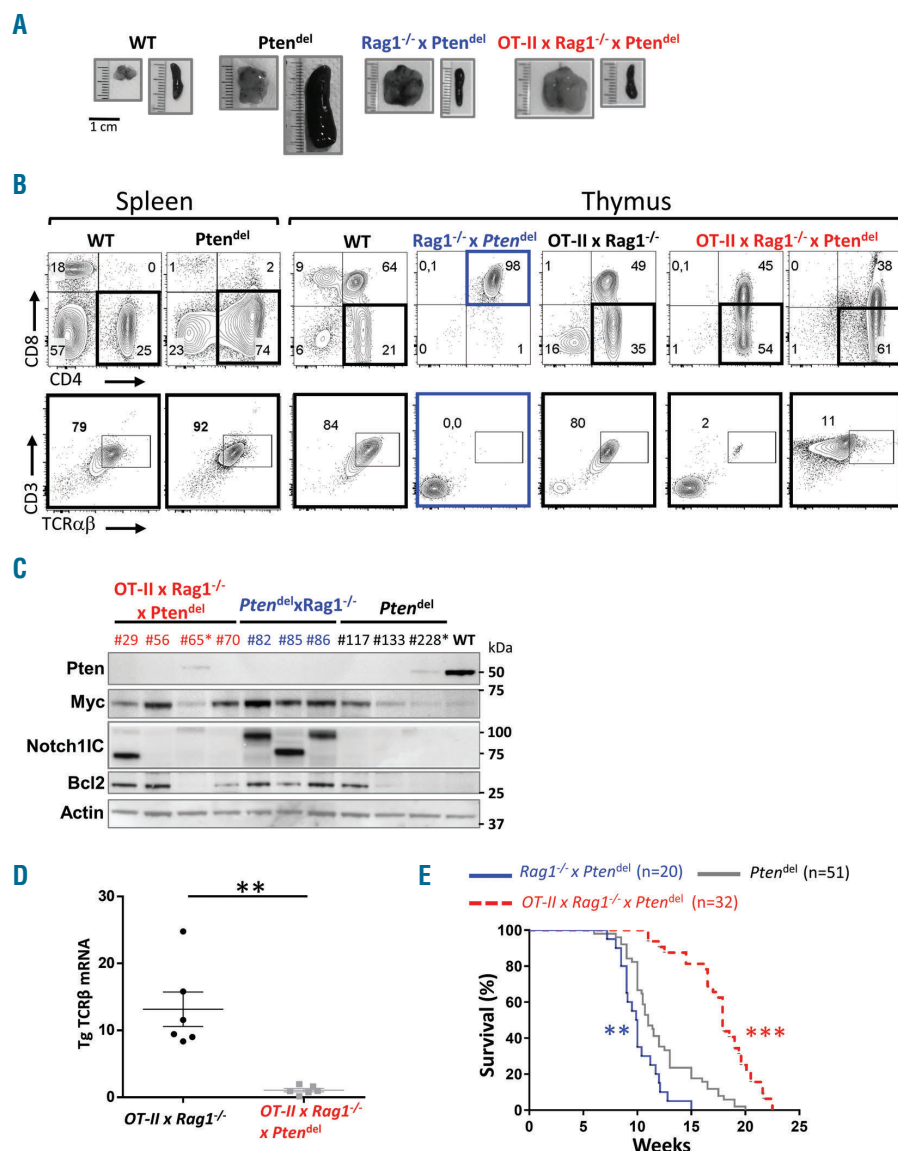


Figure 1. Fit TCR $\alpha\beta$ signaling functions as a tumor suppressor. (A) Thymus (left) and spleen (right) of typical wild-type (WT) and tumoral $Pten^{del}$, [$Rag1^{-/-} \times Pten^{del}$] and [$OT-II \times Rag1^{-/-} \times Pten^{del}$] mice. (B) Phenotypes of typical tumors generated by $Pten^{del}$ in the spleen and by [$Rag1^{-/-} \times Pten^{del}$] and [$OT-II \times Rag1^{-/-} \times Pten^{del}$] in the thymus. WT and [$OT-II \times Rag1^{-/-}$] controls are shown. CD4 SP or DP gates (top plots, bold squares) were further analyzed for CD3/TCR β expression (bottom plots). Two typical thymi of [$OT-II \times Rag1^{-/-} \times Pten^{del}$] mice are shown (1 representative of 10). (C) Thymi of indicated mice were analyzed by immunoblotting with antibodies specific for Pten, Myc, cleaved Notch1, Bcl2 and Actin as a loading control. *Identification number of analyzed mice. *Mice that did not display T-cell acute lymphoblastic leukemias (T-ALL) or T-cell lymphoblastic lymphomas (T-LBL) symptoms at the time of sacrifice. (D) Transcriptional downregulation of transgenic TCR β chain in [$OT-II \times Rag1^{-/-} \times Pten^{del}$] tumor thymocytes (n=6). Transcripts levels were normalized to ABL. Error bars show means with Standard Deviation. Statistical significance was assessed using Mann-Whitney test (** $P < 0.01$). (E) $Pten^{del}$ mice survival curve was compared to [$Rag1^{-/-} \times Pten^{del}$] or [$OT-II \times Rag1^{-/-} \times Pten^{del}$] mice survival curves using log-rank (Mantle-Cox) test (** $P < 0.01$; *** $P < 0.001$); median weeks of survival are 11 (as previously observed¹³), 9.95 and 17.9, respectively.

type or leukemic cells resuspended in RPMI medium (200 μL) and stimulated for 2 minutes (min) at 37°C with avidin (14 μg) and biotinylated anti-CD3 (10 μg; clone 2C11, BD Pharmingen, for mouse cells and clone UCHT1, eBiosciences for human cells) and biotinylated anti-CD28 (10 μg; clone 37.51, BD Pharmingen, for mouse cells and clone CD28.2, eBiosciences for human cells). Unstimulated (control) cells were incubated with avidin alone. After lysis in 2X TNE buffer (100 mM Tris, 2% Nonidet P-40, 40 mM EDTA) supplemented with protease and phosphatase inhibitors, protein extracts (approx. 60 μg) were analyzed by immunoblot (see *Online Supplementary Methods* and *Online Supplementary Table S2*). P-Tyr levels were quantified on the entire lane and normalized to ACTIN. P-AKT protein levels were normalized to AKT.

Proliferation and apoptosis assays following CD3/CD28 stimulation

For comparison of the TCR-signaling ability to mediate proliferation or apoptosis of normal or leukemic cells, 1.105 of non-purified or CD4 SP purified T cells were mixed (ratio 1:1) with Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) or Dynabeads Human T-Activator CD3/CD28 (Life Technologies) in 96-well flat bottom plates and incubated for 24 or 72 hours (h) (37°C, 5% CO₂). For unstimulated controls, thymocytes were incubated in the same conditions but without anti-CD3/CD28 coated beads. Proliferation was measured using CFSE labeling (CellTrace™ CFSE Cell Proliferation Kit, Life Technologies) and apoptosis was followed using AnnexinV labeling (BD Pharmingen) and 7-AAD (BD Pharmingen) according to the manufacturer’s instructions.

Statistical analysis

Kaplan-Meier survival curves and statistical analyses were performed using GraphPad Prism software. Survival curves were compared using log-rank (Mantle-Cox) test. Statistical significance

was evaluated by two-tailed Mann-Whitney U-test. P<0.05 was considered significant.

Further details are provided in the *Online Supplementary Methods* and antibodies used for flow cytometry are listed in *Online Supplementary Tables S3 and S4*.

Results

Pten^{del} thymocytes expressing transgenic TCR are counter-selected during leukemogenesis

Phosphatase and tensin homolog protein (PTEN) is a well-known tumor suppressor involved in numerous types of cancers, and represents the main negative regulator of PI3K/AKT signaling pathway.¹² To investigate the role of the TCR in leukemogenesis, we used a Pten-deficient mouse model of T-ALL which has previously been shown to induce TCRαβ⁺ tumors.^{13,14} Pten^{Flox/Flox} mice were crossed into a CD4-Cre background (hereafter referred to as Pten^{del}) in which Cre is fully active at the CD4⁺CD8⁺ double positive (DP) stage of thymocyte development.¹⁵ As previously described,¹⁶ Pten^{del} mice developed leukemia characterized by malignant proliferation of mono/oligo-clonal T cells (*Online Supplementary Table S5*), and enlarged thymus and spleen (Figure 1A). Peripheral leukemic blasts from Pten^{del} mice were typically CD4 SP and expressed αβTCR at their surface (Figure 1B and *Online Supplementary Table S5*), in line with previous reports.^{14,16,17} To analyze the impact of positive selection, we used the OT-II mouse model which expresses a transgenic Vα2/Vβ5.1 TCR recognizing the chicken ovalbumin antigen in the context of MHC-II molecules.¹⁸ Pten^{del} mice were crossed with OT-II Rag1-deficient mice, in which all developing T cells do express an OT-II fit TCR, designed to trigger positive selection and give rise to mature SP T

Table 1. Immuno-phenotypes of T-cell acute lymphoblastic leukemia developed by OT-II x Pten^{del} mice in I-A^{b/b} or I-A^{b/d} backgrounds.

Mouse #	I-A	CD4/CD8	TCRαβ	TCR OT-II	TCRVβ	TCRV α
25	I-A ^{b/b}	CD4 ⁺	+	Neg	Vβ14	Vα2
26	I-A ^{b/b}	CD4 ⁺ /DN	+	Neg	Vβ11	Vα2
28	I-A ^{b/b}	CD4 ⁺	+	Neg	Vβ14	Vα2
70	I-A ^{b/b}	CD4 ⁺	+	Neg	Vβ14	Vα2
83	I-A ^{b/b}	CD4 ⁺	+	Neg	Vβ14	Vα2
114	I-A ^{b/b}	CD4 ⁺	+	Neg	Vβ6	Vα2
140	I-A ^{b/b}	CD4 ⁺	+	Neg	Vβ8.1/8.2	Vα2
141	I-A ^{b/b}	CD4 ⁺ /DN	+	Neg	Vβ5	Nd*
350	I-A ^{b/b}	CD4 ⁺	+	Neg	Vβ14	Vα2
2	I-A ^{b/d}	CD4 ⁺	+	+	Vβ5	Vα2
11	I-A ^{b/d}	CD4 ⁺	+	+	Vβ5	Vα2
12	I-A ^{b/d}	CD4 ⁺	+	+	Vβ5	Vα2
17	I-A ^{b/d}	CD4 ⁺	+	Neg	Vβ6	Vα2
19	I-A ^{b/d}	CD4 ⁺	+	+	Vβ5	Vα2
278	I-A ^{b/d}	CD4 ⁺	+	+	Vβ5	Vα2
281	I-A ^{b/d}	CD4 ⁺	+	+	Vβ5	Vα2
291	I-A ^{b/d}	CD4 ⁺ /DN	+	+	Vβ5	Vα2
321	I-A ^{b/d}	CD4 ⁺ /DN	+	+	Vβ5	Vα2
354	I-A ^{b/d}	CD4 ⁺ /DP	+	+	Vβ5	Vα2

*Negativity for TCRVα2 and the TCRVα expressed was not determined.

cells. Both [OT-II x Rag1^{-/-} x Pten^{del}] and the control TCR-deficient mice [Rag1^{-/-} x Pten^{del}] (*Online Supplementary Figure S2*) developed T-cell lymphoblastic lymphomas (T-LBL) which were mostly restricted to the thymus (Figure 1A), over-expressed Bcl2¹⁹ and, as previously described,¹⁷ were recurrently Notch1-dependent (Figure 1C). Indeed, we found that all [Rag1^{-/-} x Pten^{del}] tumors tested (n=7) and 3 out of 7 [OT-II x Rag1^{-/-} x Pten^{del}] tumors

were *Notch1* activated. Of note, Notch1 activation does not impact latency of [OT-II x Rag1^{-/-} x Pten^{del}] tumors (*Online Supplementary Figure S3*). Strikingly, in the [OT-II x Rag1^{-/-} x Pten^{del}] model, the examined tumors (n=15) had lost surface expression of the OT-II transgene and were either TCR^{neg} or TCR $\alpha\beta$ ^{low} (Figure 1B), consistent with previous observations.¹⁷ Molecular analysis of the transgenic β chain mRNA expression in the tumors revealed down-

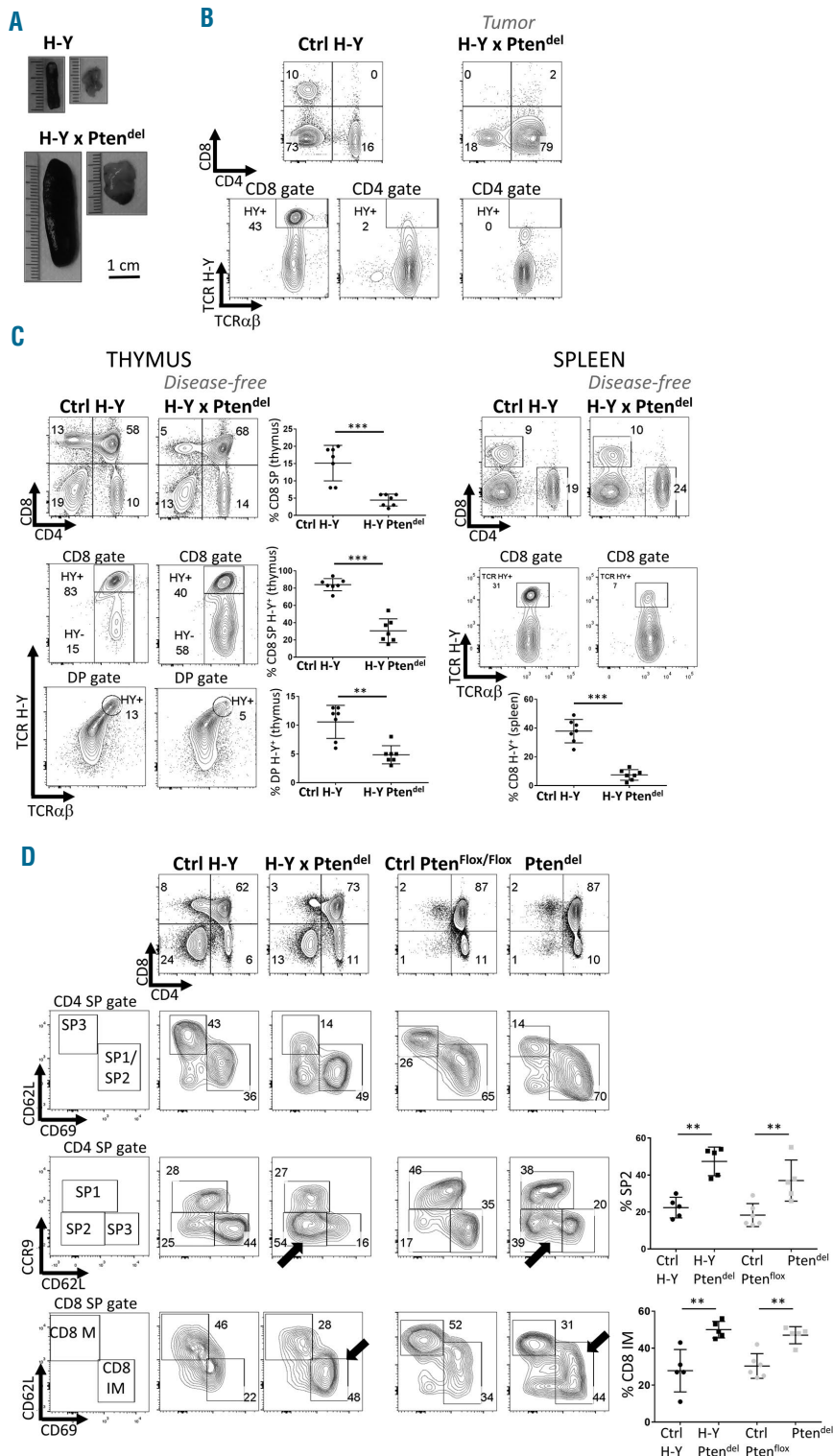


Figure 2. Counter-selection of T cells harboring H-Y TCR. (A) Spleen (left) and thymus (right) of typical H-Y and tumoral [H-Y x Pten^{del}] female mice. [H-Y x Pten^{del}] mice developed T-cell acute lymphoblastic leukemias (T-ALL) in approximately ten weeks (n=5). (B) Flow cytometry analysis of typical spleens from H-Y and tumoral [H-Y x Pten^{del}] female mice. (C) Flow cytometry analysis of typical disease-free thymus (left panels) and spleens (right panels) from young (4-week old) H-Y and [H-Y x Pten^{del}] female mice. Percentages of cells in depicted gates are indicated. Representative data of at least 3 experiments are shown. Dot plots show percentages of CD8 SP, H-Y+ CD8 SP or H-Y+ DP thymic cells and H-Y+ CD8 T cells from spleens of control H-Y (n=7) and [H-Y x Pten^{del}] (n=7) female mice. (D) Pre-tumoral single positive (SP) thymocytes are partially blocked at the immature CD69⁺CD62L^{low} stage. Analysis of 4-week old disease-free (pre-tumoral) Pten^{del} and [H-Y x Pten^{del}] mice and their respective control counterparts is shown (representative data of at least 3 experiments). CD8 M: mature CD8 SP (CD69⁺CD62L^{hi}); CD8 IM: immature CD8 SP (CD69⁺CD62L^{lo}). Arrows indicate the stage of differentiation arrest (SP2 and CD8 IM). Dot plots show percentages of CD4 SP2 and CD8 IM T cells in the indicated backgrounds (n=5 or 6). Error bars show means with Standard Deviation. Statistical significance was assessed using Mann-Whitney test (**P<0.01; ***P<0.001).

regulation of the Vβ5.1 transcript (Figure 1D). This suggests an active counter-selection of leukemic (or pre-leukemic) thymocytes bearing the transgenic TCR. The latency of tumor onset was significantly increased in [OT-II x Rag1^{-/-} x Pten^{del}] mice compared to Pten^{del} mice, consistent with the time that is likely required for selection of TCR^{neg} cells, while in the enforced absence of TCR ([Rag1^{-/-} x Pten^{del}] mice), latency was significantly reduced (Figure 1E), evoking a potential tumor suppressor role of TCR signaling in leukemogenesis.

To rule out transgenic-specific effect, we also tested the impact of selection in the H-Y mouse model, which expresses a transgenic TCR recognizing the male H-Y antigen in the context of MHC-I molecules. In female H-Y mice, negative selection is not operating, and positively selected mature H-Y⁺ TCR T cells differentiate as CD8 SP.²⁰ H-Y mice were crossed with Pten^{del} mice, on a Rag-proficient background to allow non-transgenic TCR competitive formation and development. [H-Y x Pten^{del}] females developed TCRαβ⁺ T-ALL (Figure 2A and B). Remarkably, tumors were typically CD4 SP (never CD8 SP), and none of them expressed the transgenic H-Y TCR (Figure 2B). Thus, regardless of the model used, the expression by (pre-)tumoral thymocytes of a fit TCR is counter-selected during T-ALL development. In disease-free thymi and spleens of young female [H-Y x Pten^{del}] mice (and thus before clinical tumor manifestation), we detected a severe reduction of H-Y⁺ TCR cells at the CD8 SP stage compared to control H-Y mice, and this was already apparent at the DP stage of thymocyte development (Figure 2C). In addition, this counter selection of H-Y⁺ thymocytes occurs post β-selection, after immature single positive (ISP) stage (Online Supplementary Figure S4).

These data suggest that Pten-deficient H-Y⁺ TCR thymocytes are eliminated instead of positively selected, and that this counter-selection occurs before full malignant transformation, preventing leukemia development.

Disruption of final maturation of Pten-deficient SP cells

Since most mature thymocytes with fit H-Y TCR are eliminated in young female disease-free [H-Y x Pten^{del}] mice, we analyzed the remaining H-Y TCR negative SP cells, using the CD69, CD62L and CCR9 markers of T-cell differentiation. Developmental sequence of CD4 SP thymocyte maturation is usually described as SP1 (CD69⁺, CD62L^{Low/med}, CCR9⁺), SP2 (CD69⁺, CD62L^{Low/med}, CCR9^{Neg}) and SP3 (CD69^{Neg}, CD62L^{High}, CCR9^{Neg}).^{21,22} For CD8 SP cells, the most immature cells are CD69⁺ and CD62L^{Low/med} while the more mature are CD69^{Neg} and CD62L^{High}. We observed for both [H-Y x Pten^{del}] and Pten^{del} models a partial block of positively selected cells at the immature CD69⁺CD62L^{low} stage (Figure 2D). Such differentiation arrest could provide an additional opportunity for malignant transformation.

Fit TCR signaling acts as a tumor suppressor

With the premise that Pten-deficient cells with fit TCR are counter-selected, we next assessed whether cells carrying low affinity (unfit) TCR were prone to leukemia development. OT-II TCR originates from CD4⁺ I-A^b-restricted T-cell hybridoma,¹⁸ thus positive selection is optimal in I-A^{b/b} background and sub-optimal in I-A^{b/d} background. C57BL/6 (I-A^b) [OT-II x Rag1^{-/-} x Pten^{del}] mice were crossed with BALB/C (I-A^d) mice to generate Rag-proficient [OT-II x Pten^{del}] mice on I-A^{b/d} background. In the spleens from OT-II control mice, percentages of CD4⁺ T cells dropped from approximately 78% in syngeneic I-A^{b/b} background to approximately 7% in allogenic I-A^{b/d} background (Figure 3A). [OT-II x Pten^{del}] Rag-proficient mice developed T-ALL with a similar latency as Pten^{del} mice (approx. 11 weeks), irrespective of the backgrounds (I-A^{b/b} or I-A^{b/d}). Leukemic blasts in the spleen were mostly CD4⁺ (Table 1). On the I-A^{b/b} background, while all T-ALL analyzed (n=9) expressed a TCRαβ, none of them

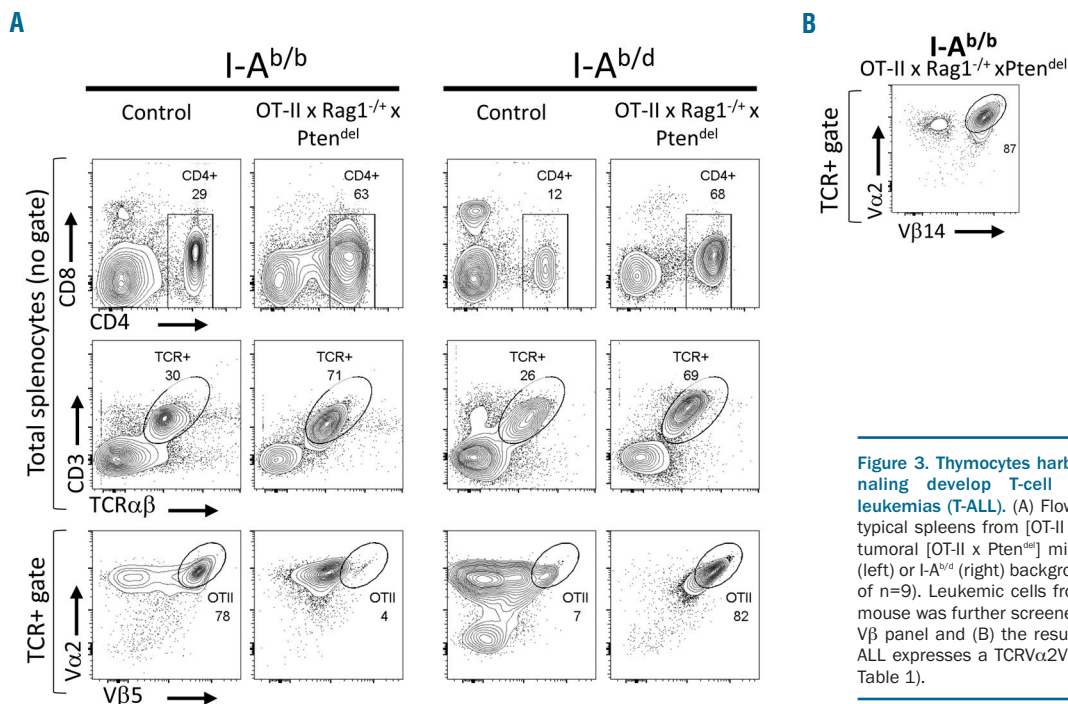


Figure 3. Thymocytes harboring unfit TCRαβ signaling develop T-cell acute lymphoblastic leukemias (T-ALL). (A) Flow cytometry analysis of typical spleens from [OT-II x Pten^{del}] (Control) and tumoral [OT-II x Pten^{del}] mice bred either on I-A^{b/b} (left) or I-A^{b/d} (right) backgrounds (1 representative of n=9). Leukemic cells from [OT-II x Pten^{del}] I-A^{b/b} mouse was further screened by cytometry using a Vβ panel and (B) the result indicates that this T-ALL expresses a TCRα2Vβ14 receptor (see also Table 1).

expressed the full transgenic OT-II TCR (usually TCRV α 2 chain was expressed, but not associated with TCRV β 5 chain) (Table 1 and Figure 3). In line with the H-Y model above, this indicates that (pre)leukemic clones harboring OT-II fit TCR are counter-selected during oncogenesis. In striking contrast, most of TCR $\alpha\beta^+$ T-ALL in the allogeneic I-A^{b/d} background (9 of 10) expressed OT-II (Table 1 and Figure 3A). This indicates that in a context of sub-optimal positive selection, Pten^{del} OT-II⁺ blasts are not counter-selected, but rather bypass death-by-neglect during posi-

tive selection allowing further leukemia development.

Altogether our data indicate that, in Pten-deficient T-ALL mouse models, fit TCR functions as a tumor suppressor impeding thymocytes to develop leukemia, while thymocytes expressing no or unfit TCR are prone to leukemogenesis.

TCR $\alpha\beta$ signaling is disabled in Pten-deficient T-ALL

We next asked whether endogenous TCR $\alpha\beta^+$ from Pten^{del} mice, which presumably passed positive selection

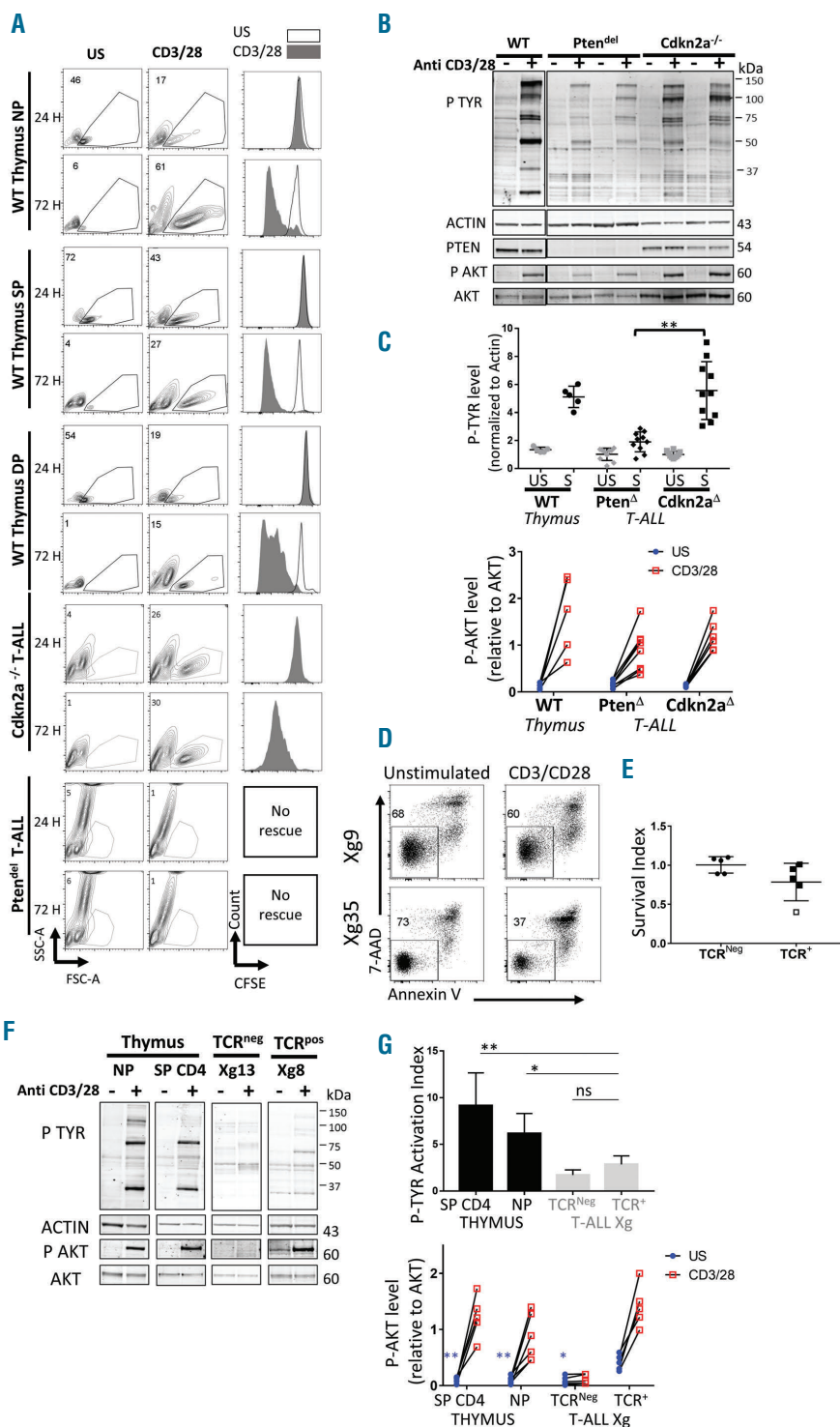


Figure 4. TCR $\alpha\beta$ signaling is disabled in Pten^{del} T-ALL blasts. (A) Indicated cells labeled with CFSE were either left unstimulated (US) or stimulated with anti-CD3/28 beads (CD3/28) for 24 or 72 hours (h), and then analyzed by flow cytometry. SSC/FSC dot plots (left) and CFSE histograms (right) are shown. Numbers indicate percentage of living cells. Representative data of Pten^{del} T-cell acute lymphoblastic leukemias (T-ALL) (n=5) and Cdkn2a^{-/-} T-ALL (n=5). (B) Analysis of early TCR signaling by immunoblots. Two representative cases of Pten^{del} T-ALL (n=5) and Cdkn2a^{-/-} T-ALL (n=5), and wild-type (WT) thymocytes are shown. Cells were untreated (-) or stimulated (+) with anti-CD3/CD28 antibodies for 2 minutes and analyzed by immunoblotting with antibodies specific for phosphorylated tyrosine (P-Tyr), phosphorylated AKT S473 (P-AKT), AKT and Actin. (C) Levels of P-Tyr species normalized to Actin (top) and of P-Akt normalized to Akt (bottom) in unstimulated (US) and in CD3/CD28-stimulated (S) of indicated cells: WT thymus, Pten^{del} T-ALL (n=5) and Cdkn2a^{-/-} T-ALL (n=5) assayed in duplicate for P-Tyr. (D and E) Impact of TCR stimulation on human T-ALL cell survival. Cells were either left unstimulated or stimulated with beads coated with anti-CD3 and anti-CD28 antibodies (CD3/CD28) during 72 h and then stained with Annexin V and 7-AAD to monitor cell death by flow cytometry. (D) Typical dot plots for Xg9 and Xg35 are shown. Percentage of live cells (gated) is indicated. (E) Survival index as determined by ratio of live cells in treated (CD3/28) versus unstimulated conditions 72 h post induction. Each dot corresponds to the mean survival index (obtained from at least 2 assays) of one T-ALL xenograft. Xg35 sample is depicted as a white square. (F and G) Human thymus and T-ALL were analyzed as described in (B). Thymus NP and SP CD4⁺ correspond to total non-purified (NP) and purified CD4 SP cells, respectively, from healthy human thymus. (F) Two representative samples of human T-ALL: Xg13 (TCR^{neg}) and Xg8 (TCR $\alpha\beta^+$) are shown (see also *Online Supplementary Figure S9*). (G) P-Tyr activation index which corresponds to the ratio of P-Tyr species levels in stimulated (CD3/28) versus unstimulated samples (top); P-AKT normalized to AKT (bottom) in unstimulated (US) and in CD3/CD28-stimulated (S) (Bottom). Statistical significance of P-AKT levels between unstimulated TCR⁺ PDX versus TCR^{neg} PDX, NP or SP thymocytes are indicated with blue asterisks. TCR^{neg} (n=5: Xg3, 13, 20, 23 & 40) and TCR $\alpha\beta^+$ (n=5: Xg8, 9, 35, 38 & 47). P-Tyr species levels were normalized to ACTIN. (C, E and G) Error bars represent means + Standard Deviation. Statistical significance was assessed using Mann-Whitney test; ns: non-significant P>0.05; *P<0.05; **P<0.01.

and developed T-ALL, acquired a deficiency in TCR signaling. Unstimulated primary mouse T-ALLs tend to quickly undergo apoptosis in liquid culture *in vitro* and can be rescued through anti-CD3/anti-CD28 TCR activation, as shown for the $\text{TCR}\alpha\beta^+$ $\text{Cdkn2a}^{-/-}$ T-ALL mouse model (Figure 4A, *Online Supplementary Table S6* and *Online Supplementary Figures S5* and *S6A*). By contrast, no rescue could be obtained for T-ALL blasts from the Pten^{del} T-ALL model, and $\text{TCR}\alpha\beta^+$ Pten^{del} leukemic cells quickly died with or without stimulation. To further investigate the impact of TCR triggering at the molecular level, freshly harvested tumors were CD3/CD28 stimulated for 2 min and analyzed by immunoblotting with antibodies specific for phosphorylated tyrosine species (P-Tyr) (Figure 4B). In control purified DP and non-purified (NP) mouse WT thymocytes, activation of the TCR pathway triggered various intracellular signaling molecules²³ leading to a marked increase in the pattern of global tyrosine phosphorylation (Figure 4B and C), as previously described.²⁴ Similar results were obtained with $\text{Cdkn2a}^{-/-}$ T-ALLs, in line with proliferation data described above. By contrast, global tyrosine phosphorylation was significantly dampened in murine $\text{TCR}\alpha\beta^+$ Pten^{del} T-ALLs (Figure 4B and C). However, P-Tyr antibody does not detect the activation of AKT which is the main downstream target of Pten.¹² Thus, we specifically monitored phosphorylation of Akt at Ser473. It was previously showed that Akt phosphorylation was very high in non-tumoral Pten-deficient thymocytes compared to Pten-proficient thymocytes.¹³ However, we found that P-Akt levels in Pten^{del} T-ALL are similar to the one detected for WT thymocytes and $\text{Cdkn2a}^{-/-}$ T-ALL (Figure 4B and C) and thus are lower than one might expect from Pten-deficient thymocytes.¹³

It is noteworthy that when we induced inactivation of Pten in $\text{Cdkn2a}^{-/-}$ T-ALL cells, the ability of those cells to proliferate upon stimulation was conserved (*Online Supplementary Figure S7*), suggesting that once pre-tumoral thymocytes have passed selection and the tumor is established, late deletion of Pten no longer interferes with TCR-mediated activation. In the same line, T cells from disease-free Pten^{del} spleen were able to proliferate upon anti-CD3/28 stimulation (*Online Supplementary Figure S8*), confirming that, *per se*, Pten loss is not directly responsible for the TCR signaling inhibition observed in Pten^{del} T-ALL. Together with the fact that human late cortical T-ALLs are frequently carrying PTEN loss-of-function alterations,⁵ this pointed to a possible role of Pten loss in the dysregulation of the selection process. We thus assessed whether the above observation indicating that TCR signaling is impaired in Pten-deficient T-ALL was relevant in human primary T-ALL samples. To obtain adequate quantities of viable human leukemia cells devoid of contaminating residual physiological mature T cells, samples from T-ALL patients were engrafted into immunodeficient NSG mice. In our patient-derived xenograft (PDX) collection, PTEN was present in all TCR^{neg} T-ALL, while it was not expressed in 5 out of 6 $\text{TCR}\alpha\beta^+$ samples. We investigated the 5 PTEN-deficient $\text{TCR}\alpha\beta^+$ T-ALL (T-ALL 8, 9, 35, 38 and 47; their corresponding xenografts were denoted Xg8, Xg9, Xg35, Xg38 and Xg47), and 5 TCR^{neg} T-ALLs were used as controls (Xg3, Xg13, Xg20, Xg23 and Xg40) (*Online Supplementary Table S7* and *Online Supplementary Figure S6B*). Leukemic grafts were harvested from mice and stimulated with anti-CD3 and anti-CD28. In contrast to mouse, human T-ALL do not die quickly in liquid cul-

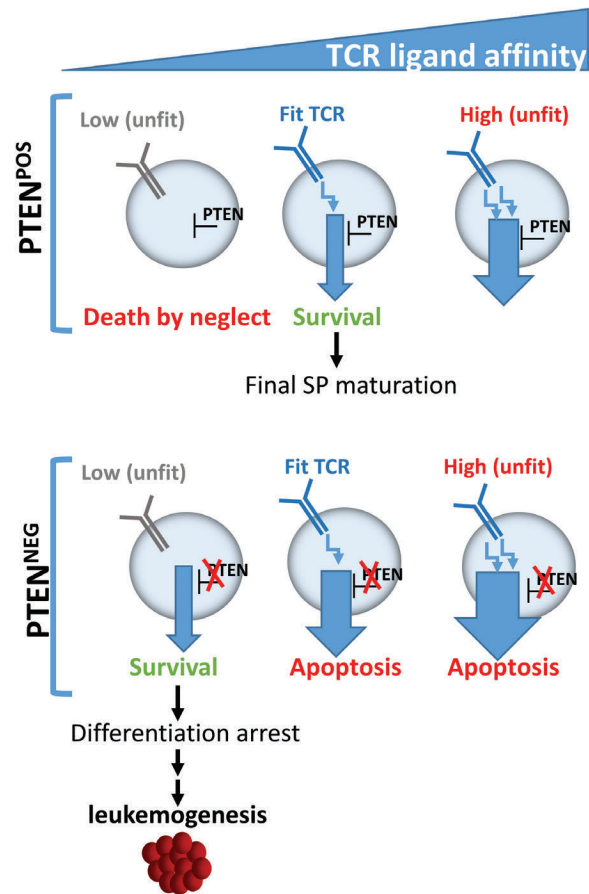


Figure 5. Model for integration of Pten loss-of-function and TCR signaling-mediated tumor suppression. In the context of Pten loss, thymocytes bearing fit or high affinity TCR would be eliminated while those bearing no/low affinity TCR would be rescued from death-by-neglect. However, harboring TCR complex that does not signal properly would prevent further thymocyte differentiation, providing an additional opportunity for malignant transformation.

ture; thus we assessed the impact of TCR stimulation at the cellular level. We observed that activation-induced cell death (AICD) was triggered for only 1 $\text{TCR}\alpha\beta^+$ T-ALL (Xg35), the remaining 4 $\text{TCR}\alpha\beta^+$ T-ALL, as well as control TCR^{neg} T-ALLs, being resistant to AICD (Figure 4D and E). To investigate signaling downstream of the TCR, as described above for mouse T-ALL, PDX cells were lysed 2 min post activation with anti-CD3/CD28, and analyzed by immunoblotting. In control (disease-free) non-purified (NP) or purified CD4 SP human thymocytes, activation of the TCR pathway led to a marked increase in the pattern of global tyrosine phosphorylation. In contrast, the $\text{TCR}\alpha\beta^+$ T-ALLs samples showed a reduced and somewhat intermediate activation of tyrosine phosphorylated species compared to NP or CD4 SP thymocytes and $\text{TCR}\alpha\beta^{\text{neg}}$ controls (Figure 4F). Globally, the P-Tyr activation index of $\text{TCR}\alpha\beta^+$ T-ALL was significantly lower than physiological controls (NP or CD4 SP; $P < 0.05$) but not significantly higher than $\text{TCR}\alpha\beta^{\text{neg}}$ T-ALL (Figure 4G). In contrast, AKT phosphorylation was detected in PTEN-deficient $\text{TCR}\alpha\beta^+$ T-ALLs in unstimulated conditions, then following stimulation, it was slightly increased (approx. 3.5-fold) and ended up equivalent to CD4 SP positive control (Figure 4G).

Taken together, these data indicate that both in mouse and human, TCR $\alpha\beta$ signaling network is largely disabled in PTEN-deficient TCR $\alpha\beta$ ⁺ T-ALLs.

Discussion

Here we undertook to investigate the impact of TCR signaling during T-ALL leukemogenesis. We show that in a mouse model of Pten loss-of-function, a frequent event among human TCR $\alpha\beta$ ⁺ T-ALLs (approx. 70%) (*Online Supplementary Table S8*), early counter-selection of fit TCR $\alpha\beta$ ⁺ thymocytes, occurs before the onset of leukemia development. Furthermore, we show that established TCR $\alpha\beta$ ⁺ T-ALLs are carrying TCRs which are unfit and/or impaired in signaling.

T-ALLs represent the malignant counterparts of most thymocyte stages of development. Analysis of T-ALL subtype distribution based on a human T-ALL cohort of 230 subjects²⁵ showed that early immature (n=52), cortical T-ALL (n=103), mature TCR $\gamma\delta$ ⁺ T-ALL (n=36) and mature TCR $\alpha\beta$ ⁺ T-ALL (n=39) represent 22.6%, 44.8%, 15.6% and 17%, respectively, of T-ALL cases. Intriguingly, among the TCR⁺ T-ALL, TCR $\alpha\beta$ ⁺ T-ALLs were under-represented (52%) relative to TCR $\gamma\delta$ ⁺ (48%) compared to physiological counterparts in which TCR $\alpha\beta$ largely dominate the fraction of TCR⁺ thymocytes (approx. 95% vs. approx. 5% TCR $\gamma\delta$).²⁶ Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells are not restrained to MHC and do not undergo conventional MHC-mediated positive and negative selections.^{26,27} Conversely to pre-tumoral $\alpha\beta$ T cells, pre-tumoral $\gamma\delta$ T cells might thus not be counter-selected, possibly explaining their over-representation compared to TCR $\alpha\beta$ ⁺ T-ALL.

Our *in vivo* data showed that pre-tumoral Pten^{del} cells with unfit TCR signaling (OT-II in I-A^{b/d} background) are positively selected for leukemogenesis, while thymocytes with fit TCR signaling (H-Y or OT-II in I-A^{b/b} background) are counter-selected and never developed leukemia. Thus this study points to a role of Pten during the positive selection process. Yet the specific molecular mechanism allowing positive selection of pre-tumoral cells with unfit TCR (and counter-selection of cells with fit TCR) in the absence of Pten remains to be determined. A possible scenario would be that Pten loss merely shifts the window of positive and negative selection thresholds (Figure 5). On one hand, PTEN loss might substitute for missing TCR signaling, allowing cells with no TCR or low affinity TCR to be rescued from death by neglect and bypass positive selection. Accordingly, an increase in positively selected T cells was observed in mouse models in which AKT was hyperactive.²⁸ Herein, we found that, following TCR-stimulation, AKT activation is similar in Pten-deficient T-ALL and in WT thymocytes. Therefore, while most of the TCR signaling network is disabled (Figure 4), AKT pathway appears 'normal' and, in the context of our scenario (Figure 5), is likely to be the main element contributing to the bypass of positive selection by Pten-deficient thymocytes harboring unfit/low TCR. On the other hand, integration of signals resulting from both PTEN loss and a fit TCR (passing positive selection) might reach over-threshold signaling and trigger negative selection, eliminating thymocytes carrying fit TCRs even before malignant transformation. This would involve multiple pathways downstream of PTEN loss,^{12,29} since a mere AKT

hyperactivation was insufficient to recapitulate the loss of fit H-Y⁺ thymocytes.²⁸ A challenging perspective would be to decipher the molecular mechanism underlying the counter-selection of Pten-deficient fit $\alpha\beta$ TCR⁺ thymocytes and then to assess the possibility of activating this apoptotic program in tumoral cells.

Our data indicate that TCR $\alpha\beta$ signaling pathway is actively involved in T-ALL oncogenesis. We show that TCR $\alpha\beta$ signaling can impede the development of Pten-deficient tumors and thus acts as a *bona fide* tumor suppressor. However, given the diametrically opposed effect of TCR activation on discrete stages of T-cell development, the TCR might also have pro-oncogenic effects in other contexts and/or developmental stages. For example, we show that in a Pten-proficient Cdkn2a^{-/-} T-ALL model, TCR $\alpha\beta$ ⁺ tumors are sensitive to TCR activation. Likewise, thymocytes harboring fit H-Y TCR are not counter-selected in female *TEL-JAK2* mouse model and develop leukemia.⁸ In the same line, Pten-deficient TCR α ^{-/-} or SLP76^{-/-} mice, in which TCR signaling is abrogated, display delayed tumor onset.¹⁷ Yet, and in contrast to the Pten^{del} model, TCR α ^{-/-} and SLP76^{-/-} thymocytes are blocked before (and therefore not subjected to) positive selection.^{30,31} Pre-TCR might also be directly involved in oncogenesis. For instance, in dominant active *NOTCH1* (ICN1) model, pre-TCR signaling is required for tumorigenesis.³² Conversely [Rag1^{-/-} x Pten^{del}] thymocytes that are devoid of pre-TCR bypass β -selection and develop DP T-cell lymphoma in short latency (Figure 1); this is also in striking contrast to Pten-deficient TCR α ^{-/-} or SLP76^{-/-} thymocytes (described above) that express a pre-TCR and for which leukemogenesis is impaired.¹⁷ In normal β -selected cells, the exit of proliferation is induced by pre-TCR signals that inhibit *Notch1* pathway leading to Myc downregulation.^{33,34} In [Rag1^{-/-} x Pten^{del}] DP lymphoma, Notch1 pathway is systematically activated leading to sustained expression of Myc (Figure 1C) it might be that pre-TCR signaling exerts a tumor suppressor role by shutting down Notch1 and Myc pathways.

A recent study indicated that TCR $\alpha\beta$ ⁺ T-ALL were prone to activation-induced cell death (AICD), and anti-CD3 stimulation of TCR signaling was proposed as a therapeutic strategy to eliminate leukemic blasts.⁹ By contrast, most of our TCR $\alpha\beta$ ⁺ T-ALL samples (4 of 5) were resistant to AICD (Figure 4E). This discrepancy could be due to the stage of arrest (before or after positive/negative selections) of T-ALL samples, as in both studies sensitive TCR $\alpha\beta$ ⁺ samples (4 of 5 in the *in vivo* analysis of Trinquand *et al.*⁹ and 1 of 5 in our study) were CD1a⁺, consistent with an arrest at cortical DP stage, during which positive and negative selections occur.³⁵ In contrast, AICD-resistant samples in both studies represented true late-cortical CD1a^{neg} SP T-ALLs. This cautions that anti-CD3 therapeutic strategies might be restrained to a subgroup of sensitive TCR⁺ T-ALL (such as TCR $\alpha\beta$ ⁺CD1a⁺, eventually the rare TCR $\alpha\beta$ ⁺PTEN⁺ cases, or TCR $\gamma\delta$ ⁺⁹), and thus for mature TCR $\alpha\beta$ ⁺PTEN^{neg} T-ALL alternative options should be considered. Here we have showed that integration of Pten loss and fit TCR signaling promotes a deletional program. Thus, an attractive perspective would be to decipher the mechanism underlying this apoptotic program in order to uncover an actionable target inducing cell-death, which might open new therapeutic avenues for this poor prognosis PTEN-deficient TCR $\alpha\beta$ ⁺ subgroup.³⁶

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