

Silva et al. “PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability”

**Supplementary Table; Supplementary Figures and legends S1-S21;
Supplementary Materials and Methods**

Table S1. Primers used for PTEN amplification and sequencing

Exon	Forward Primer	Reverse Primer
1	AGTCGCTGCAACCATCCA	TCTAAGAGAGTGACAGAAAGGTA
2	TGACCACCTTTTATTACTCCA	TAGTATCTTTTTCTGTGGCTTA
3	ATAGAAGGGGTATTTGTTGGA	TCCTCACTCTAACAAGCAGATA
4	TTCAGGCAATGTTTGTTA	TTCGATAATCTGGATGACTCA
5	ACCTGTTAAGTTTGTATGCAAC	TCCAGGAAGAGGAAAGGAAA
6	CATAGCAATTTAGTGAAATAACT	GATATGGTTAAGAAAAGTGTTC
7	TGACAGTTTGACAGTTAAAGG	GGATATTTCTCCCAATGAAAG
8	CTCAGATTGCCTTATAATAGTC	TCATGTTACTGCTACGTAAAC
9	AAGGCCTCTTAAAAGATCATG	TTTTCATGGTGTTTTATCCCTC

Forward and reverse primers used for PCR amplification and sequencing of each indicated PTEN exon. One additional primer for sequence analysis of the reverse strand of exon 8 was also synthesized: AACTTGTC AAGCAAGTTCTTC.

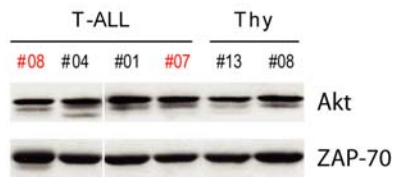


Figure S1. Akt levels are similar in primary T-ALL samples with or without activation of PI3K/Akt signaling pathway and in normal thymocytes. (A) Primary T-ALL cells and thymocytes were analyzed for Akt expression by immunoblot. Patient samples indicated in red (T-ALL#07 and T-ALL#08) display low levels of PI3K/Akt signaling pathway activation, comparable to those of normal thymocytes (Figure 5E and Table 1), whereas samples in black (T-ALL#01 and T-ALL#04) show PI3K/Akt pathway hyperactivation (Figure 1A).

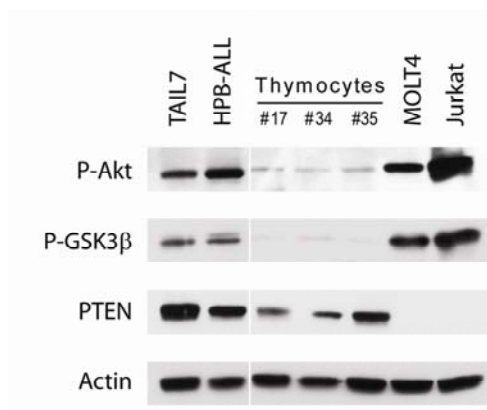


Figure S2. Hyperactivation of PI3K/Akt pathway in T-ALL cell lines. Cell lysates of normal human thymocytes or PTEN-positive (TAIL7, HPB-ALL) and PTEN-negative (MOLT4, Jurkat) T-ALL cell lines were analyzed by immunoblotting with the indicated antibodies.

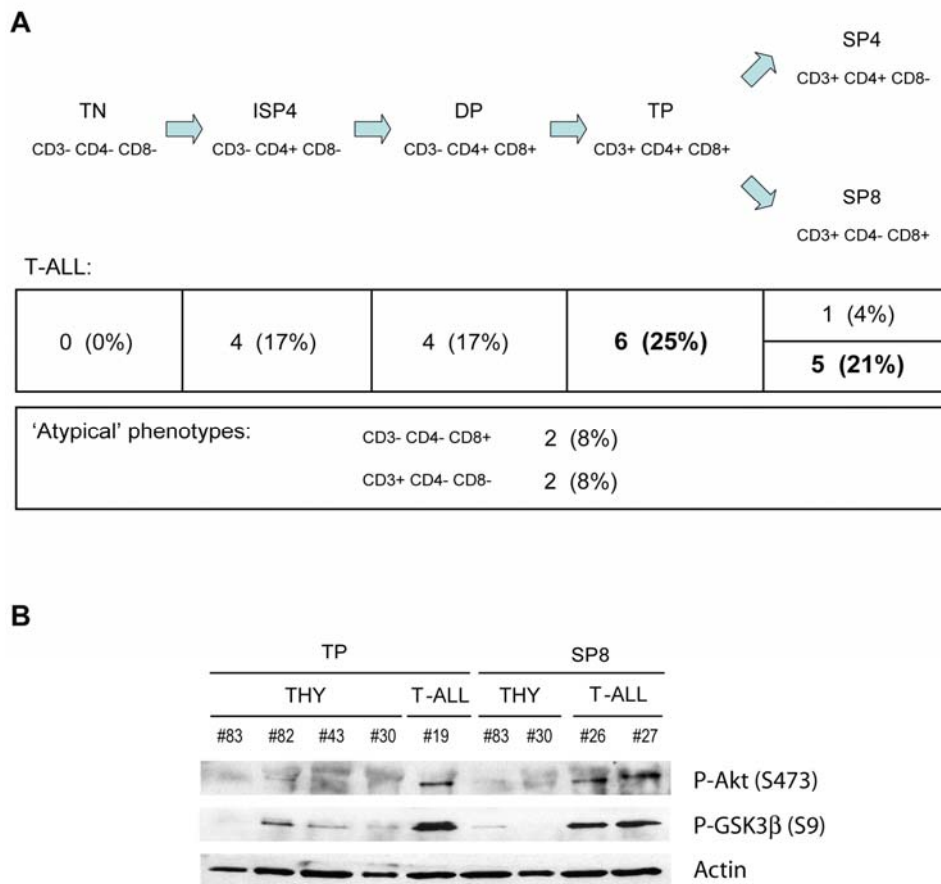


Figure S3. Isolated normal T-cell precursor subpopulations display lower levels of PI3K/Akt signaling pathway activation than their malignant immunophenotypical equivalents. (A) Simplified scheme of normal T-cell development in humans and total number and percentage of primary T-ALL cases analyzed with corresponding immunophenotype. The most common T-ALL phenotypes are indicated in bold (TP and SP8). (B) Normal thymocytes were stained for CD3, CD4 and CD8, and CD3+CD4+CD8+ (TP), and CD3+CD4-CD8+ (SP8) subpopulations were isolated by FACS (TP: >83%; SP8: >96% purity) and immediately lysed for immunoblot analysis of phosphorylated Akt and GSK3β. Primary T-ALL samples with equivalent immunophenotype were subjected to the same procedure.

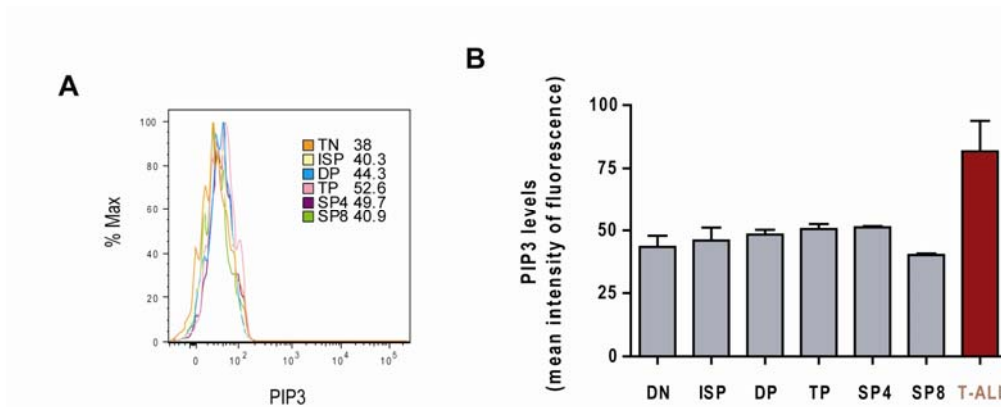


Figure S4. Major normal T-cell precursor subpopulations exhibit similar PIP3 levels that are lower than those of primary T-ALL cells. (A) Normal thymocytes were stained for CD3, CD4 and CD8 and subsequently assessed for PIP3 levels using an anti-PIP3-specific antibody by flow cytometry analysis within the gated subpopulations: TN, CD3-CD4-CD8-; ISP, CD3-CD4+CD8-; DP, CD3-CD4+CD8+; TP, CD3+CD4+CD8+; SP4, CD3+CD4+CD8-; SP8, CD3+CD4-CD8+. One representative thymus out of six analyzed is shown. Values indicate PIP3 mean intensity of fluorescence for each subpopulation. **(B)** Four primary T-ALL samples were subjected to the same procedure and analyzed at the same time. T-ALL were ISP (n=2), TP (n=1) and SP8 (n=1). Columns indicate average PIP3 MIF \pm sem for each thymic subpopulation (n=5) and T-ALL cells (n=4). One-way ANOVA statistical analysis with Bonferroni's correction showed that none of the thymic subpopulations differed from each other ($P > 0.05$ for all comparisons), but each and every normal thymic subset was significantly different from the T-ALL group ($P < 0.001$ for DN, ISP or SP8 vs T-ALL; $P < 0.01$ for DP, TP or SP4 vs T-ALL).

ALL cells (panel E in this figure, Figure 2A, and data not shown). Values indicate PTEN mean intensity of fluorescence (MIF). **(C)** Normal thymocytes were stained for CD4 and CD8 and the four major thymic T-cell precursor subpopulations evaluated for PTEN protein expression by flow cytometry analysis. PTEN expression within gated CD4-CD8- (DN), CD4+CD8+ (DP), CD4+CD8- (SP4) and CD4-CD8+ (SP8) subpopulations is indicated by different histogram colors. Corresponding negative controls (secondary antibody only) are denoted by the same colors (lighter histograms). Values indicate PTEN MIF within each subpopulation. One representative thymus (THY#24) is shown out of three analyzed. **(D)** PTEN expression within each subpopulation was analyzed in three different thymic samples. Differences are not statistically significant ($P=0.57$; One-way ANOVA). TAIL7 and Jurkat PTEN levels are represented as references. **(E)** To ensure that TAIL7 cells are representative of PTEN-positive primary T-ALL samples, PTEN expression was evaluated by densitometry analysis after immunoblotting in T-ALL primary cells and cell lines, including TAIL7. Black columns: PTEN-negative T-ALL cells; Red columns: PTEN-positive T-ALL cells. **(F)** A second thymic sample (THY#26) is depicted to further illustrate that each thymic subpopulation has higher PTEN levels than PTEN-null Jurkat T-ALL cells and lower levels than PTEN-positive TAIL7 T-ALL cells. Values indicate PTEN MIF in each thymocyte subpopulation. Jurkat and TAIL7 histograms are the same as in panels A and B, respectively, and are overlaid with each thymocyte subpopulation to simplify visual comparison. **(G)** Immunoblot analysis of PTEN expression in CD3+CD4+CD8+ TP and CD3+CD4-CD8+ SP8 T-ALL cells and sorted thymocytes. Actin was used as a loading control.

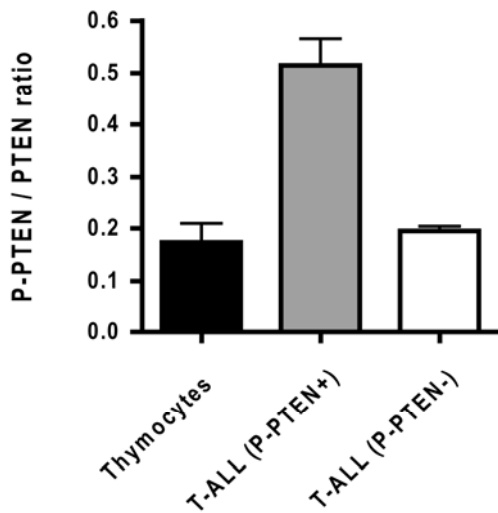


Figure S6. T-ALL cells displaying high levels of phosphorylated PTEN have increased phospho-PTEN / total PTEN ratio as compared to normal thymocytes. Levels of phosphorylated PTEN (S380) and total PTEN were assessed by immunoblot and quantified by densitometry analysis in normal thymocytes (n=8), T-ALL primary cells displaying high phospho-PTEN (P-PTEN+, n=10) and T-ALL primary cells displaying normal levels of phospho-PTEN (P-PTEN-, n=2). Columns and error bars indicate the mean \pm sem ratio of phosphorylated PTEN over total PTEN (P-PTEN / PTEN). One-way ANOVA statistical analysis with Bonferroni's correction showed that P-PTEN+ T-ALL cells presented P-PTEN/PTEN ratios that were significantly higher than those of normal thymocytes ($P < 0.001$) or P-PTEN- T-ALL cells ($P < 0.05$). In contrast, P-PTEN/PTEN ratios did not differ significantly in normal thymocytes versus P-PTEN- T-ALL cells ($P > 0.05$).

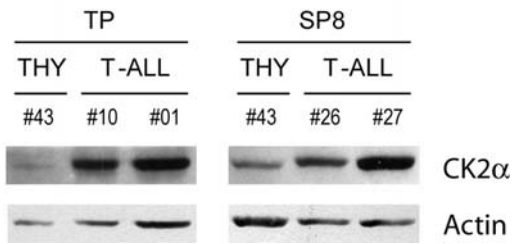


Figure S7. Isolated normal T-cell precursor subpopulations express lower CK2 protein levels than their malignant immunophenotypical equivalents. Normal thymocytes were stained for CD3, CD4 and CD8, and CD3+CD4+CD8+ (TP), and CD3+CD4-CD8+ (SP8) subpopulations were isolated by FACS (TP: 83%; SP8: 97% purity) and immediately lysed for immunoblot analysis of CK2α. Primary T-ALL samples with equivalent immunophenotype were subjected to the same procedure.

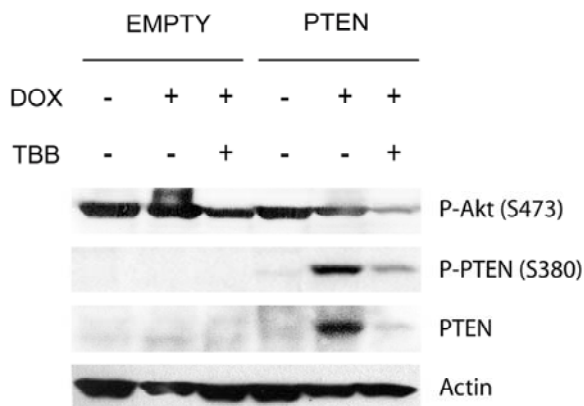


Figure S8. Downregulation of Akt phosphorylation upon CK2 inhibition using TBB is largely dependent on PTEN expression. PTEN-negative T-ALL Jurkat cells stably transfected with either PTEN or the empty vector containing a tetracycline response element were cultured with or without doxycycline for 24h and subsequently incubated for 2h30 in the presence or absence of 25μM TBB. Cells were then lysed and assessed for phospho-Akt, phospho-PTEN and PTEN by immunoblot.

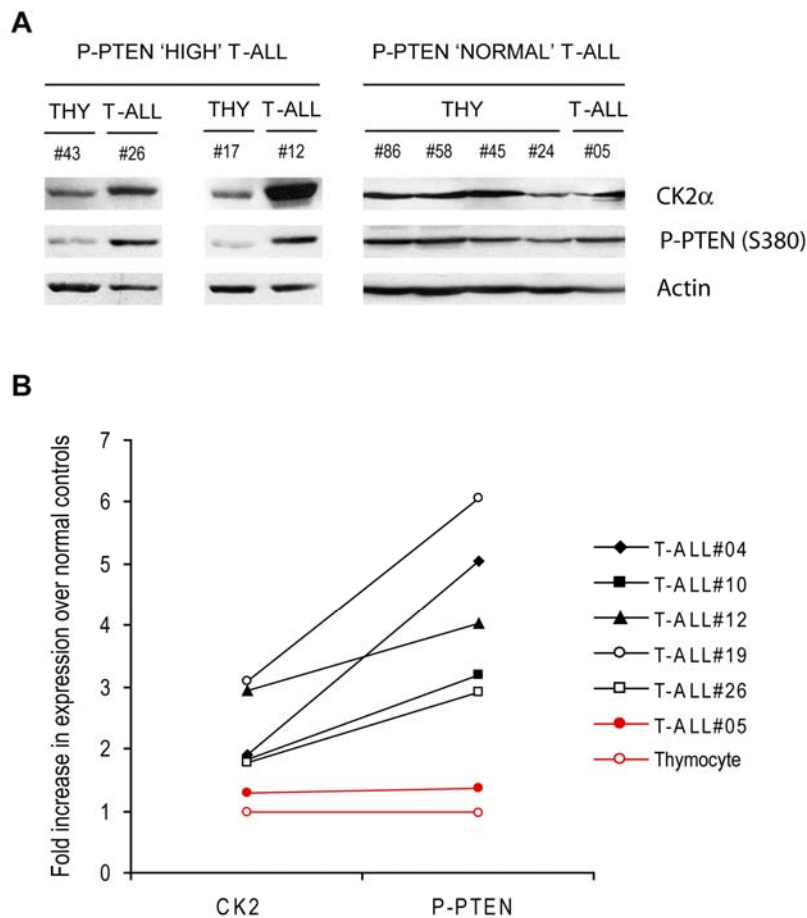


Figure S9. PTEN phosphorylation correlates with CK2 expression in PTEN-positive primary T-ALL cells. (A) Expression of CK2 α and phosphorylated PTEN (S380) was analyzed by immunoblot in two T-ALL cases with high phospho-PTEN levels (T-ALL#12 and #26) and one with phospho-PTEN levels similar to normal thymocytes (T-ALL#05). The two primary T-ALL cases with high phospho-PTEN levels overexpressed CK2, whereas T-ALL#05 displayed CK2 levels that are similar to normal controls. Blots concerning T-ALL#26 are from the same analysis presented in Figure S7. (B) Immunoblot analysis of CK2 α and phospho-PTEN (S380) in T-ALL was quantified by densitometry, normalized to actin loading control and expressed as fold-increase in expression as compared to normal control thymocytes. T-ALLs# 04, 10, 12, 19 and 26 presented fold increases ranging from 1.79 to 3.09 (CK2 α) and from 2.92 to 6.06 (P-PTEN); whereas T-ALL#05 showed similar levels to normal thymocytes (<1.5 fold increase): 1.28 (CK2 α) and from 1.38 (P-PTEN).

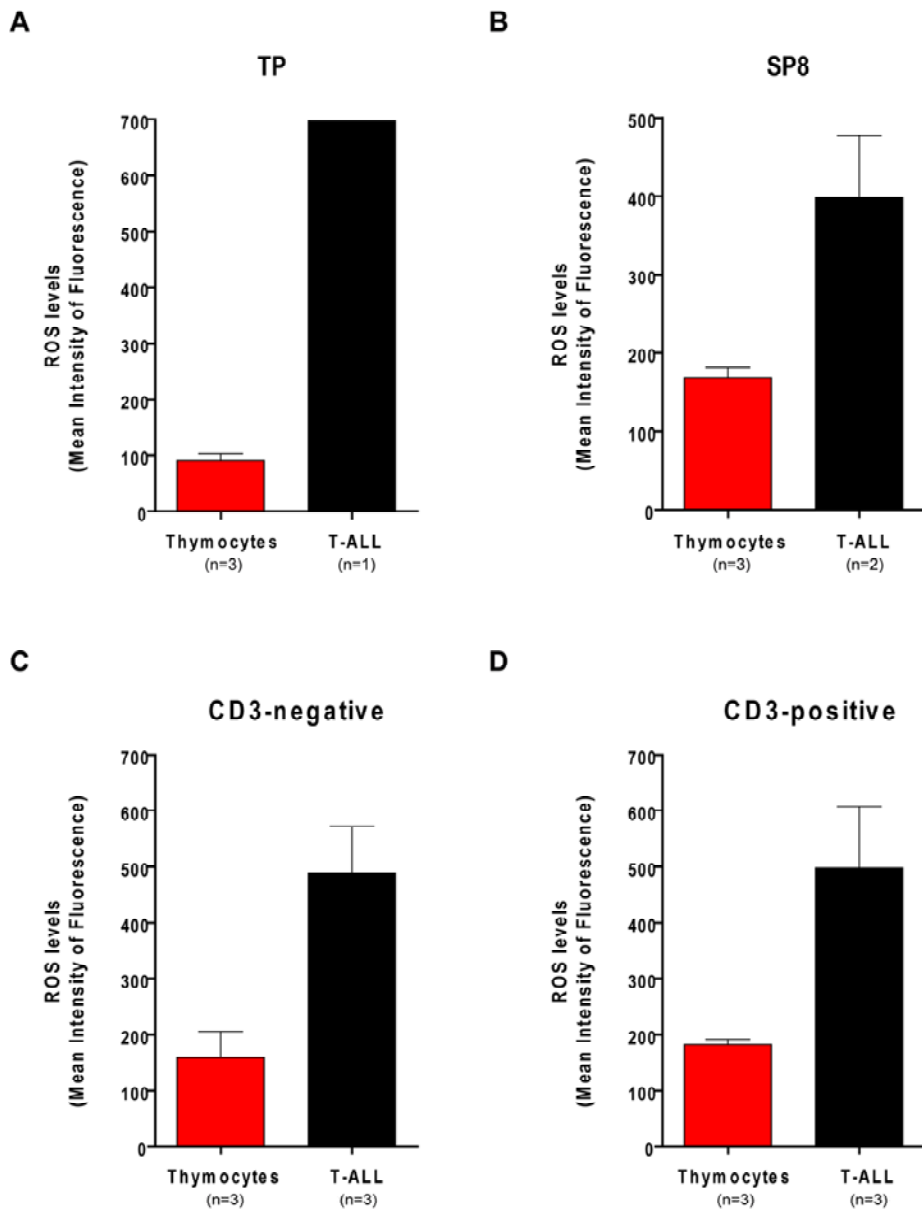


Figure S10. Normal T-cell precursor subpopulations exhibit lower ROS levels than primary T-ALL cells with equivalent immunophenotype. Normal thymocytes were stained for CD3, CD4 and CD8 and subsequently assessed for ROS levels with DCF-DA by flow cytometry analysis within gated (A) CD3+CD4+CD8+ (TP), (B) CD3+CD4-CD8+ (SP8), (C) CD3-negative, or (D) CD3-positive subpopulations. Primary T-ALL cells with the equivalent immunophenotypes were subjected to the same procedure and analyzed at the same time. Columns indicate average DCF-DA MIF \pm sem for each thymic subpopulation and corresponding T-ALL cells.

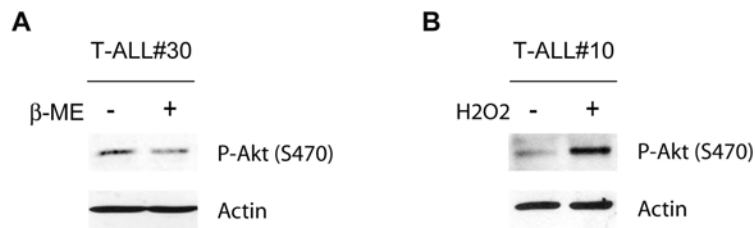


Figure S11. Analysis of modulation of PI3K/Akt signaling by ROS manipulation in additional primary T-ALL samples. PTEN-positive primary T-ALL cells were treated for 2h with 0.5 mM β-ME (**A**) or 30 min. with 1 mM H₂O₂ (**B**) and levels of phospho-Akt were determined by immunoblot.

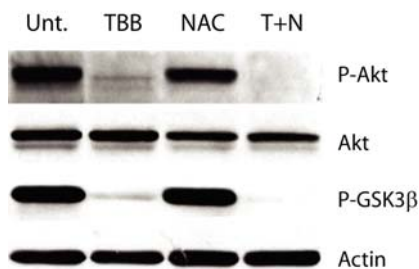


Figure S12. Combination of low doses of the CK2 inhibitor TBB and the antioxidant NAC cooperate in inhibiting PI3K/Akt pathway activation. TAIL7 cells were treated for 2h30min with 12.5μM TBB, 4mM NAC, or a combination of the two (T+N), and analyzed by immunoblotting.

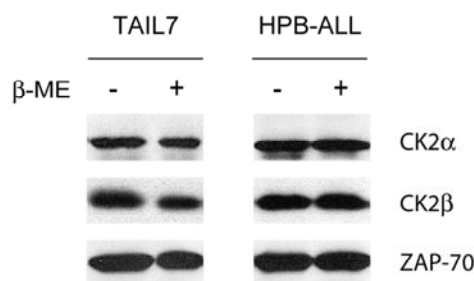


Figure S13. ROS scavenging using β-ME does not significantly affect CK2 levels in T-ALL cells. T-ALL cell lines TAIL7 and HPB-ALL were treated with 1mM β-ME for 24h. Expression of CK2α and CK2β was evaluated by immunoblot.

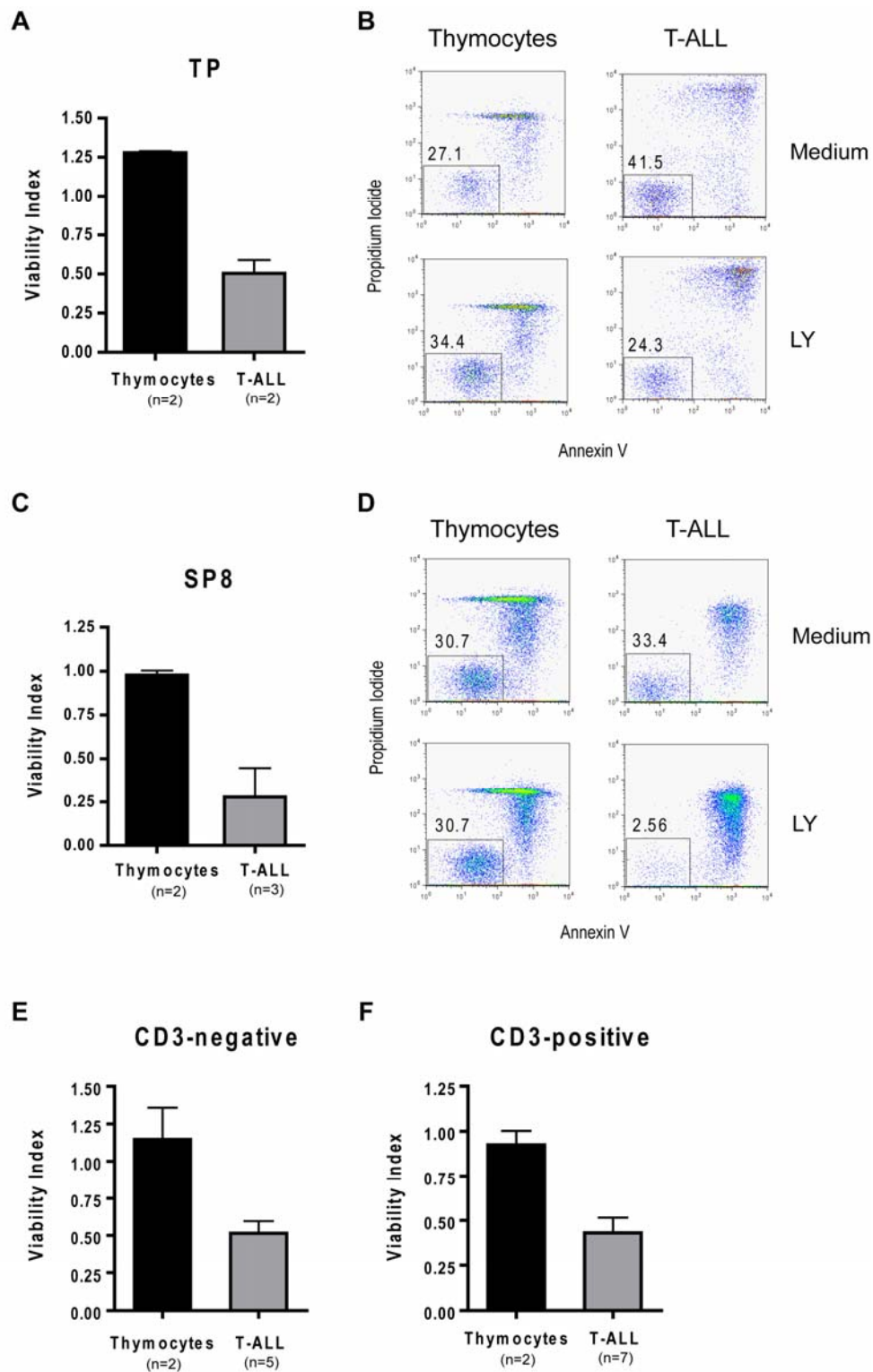


Figure S14. Isolated normal T-cell precursor subpopulations are not sensitive to PI3K pharmacological inhibition, in contrast to their malignant immunophenotypical equivalents. Normal thymocytes were stained for CD3, CD4 and CD8, and (A,B) CD3+CD4+CD8+ (TP), (C,D) CD3+CD4-CD8+ (SP8) subpopulations were isolated by FACS (TP: >83%; SP8: >90% purity) and cultured for

24h in medium alone or with 25 μ M LY294002 (LY). Primary T-ALL samples with the corresponding immunophenotype were cultured under the same conditions. **(A,C)** Mean (\pm sem) viability index to control untreated cells. **(B,D)** Representative AnnexinV / PI dot plots. Values indicate percent live cells. Similar results were obtained after 48h of culture and also using 10 μ M LY294002 (not shown). **(E,F)** Normal CD3- and CD3+ thymocytes were isolated by FACS (both populations: >98% purity) and cultured for 24h in medium alone or with 25 μ M LY294002. Primary CD3-negative and CD3-positive T-ALL samples (immature and mature T-ALL specimens, respectively, according to the classification by Uckun et al. – see Table 1) were cultured under the same conditions. Mean (\pm sem) viability index of CD3-negative **(E)** and CD3-positive **(F)** LY-treated to control untreated cells is indicated.

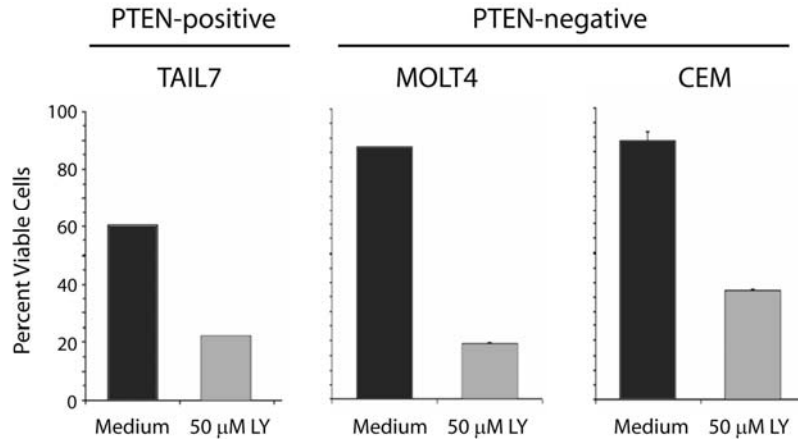


Figure S15. Inhibition of PI3K induces cell death of both PTEN-negative and PTEN-positive T-ALL cell lines. T-ALL cell lines were cultured for 48h (TAIL7; PTEN-positive) or 24h (MOLT4, CEM; PTEN-negative) in the presence or absence of LY (50μM) and percent viability was determined by flow cytometry analysis. Columns and error bars indicate mean ± sem of triplicates.

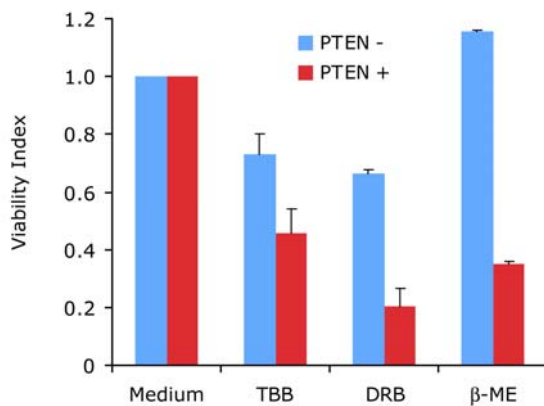


Figure S16. Effect of CK2 inhibition or ROS scavenging on viability of T-ALL cells is largely dependent on PTEN expression. PTEN-negative T-ALL Jurkat cells stably transfected with a vector containing a tetracycline response element driving the expression of PTEN were cultured with (PTEN +) or without (PTEN -) doxycycline for 24h and subsequently cultured in medium or with 40μM TBB, 25μM DRB or 0.1mM β-ME for 48h. Empty vector Tet-on T-ALL Jurkat cells responded to TBB, DRB and ROS similarly to PTEN – cells and independently of being treated with or without doxycycline (not shown).

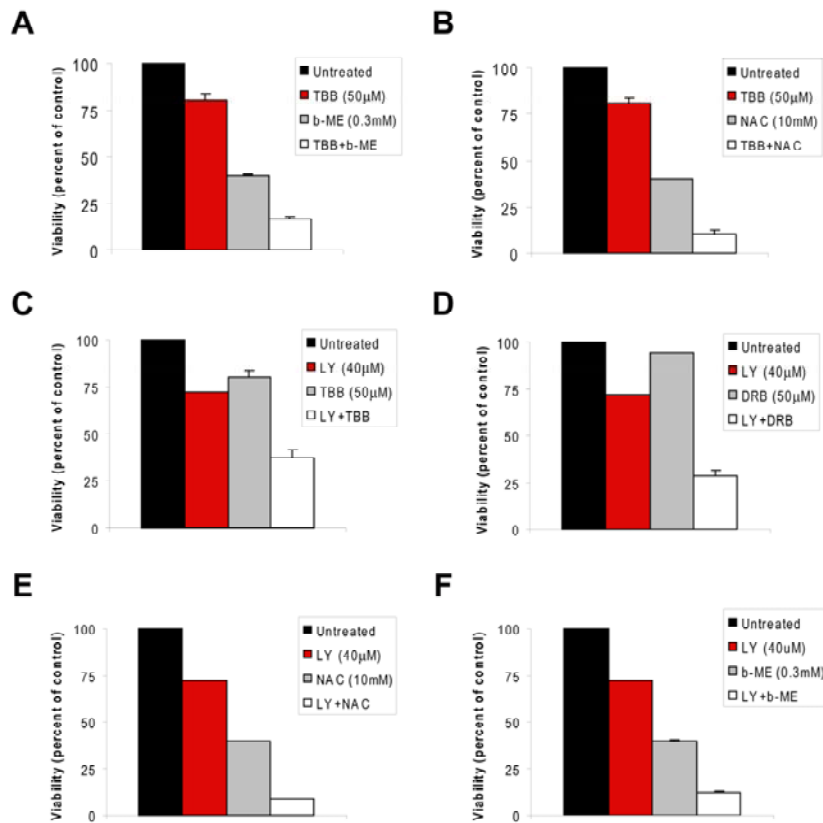
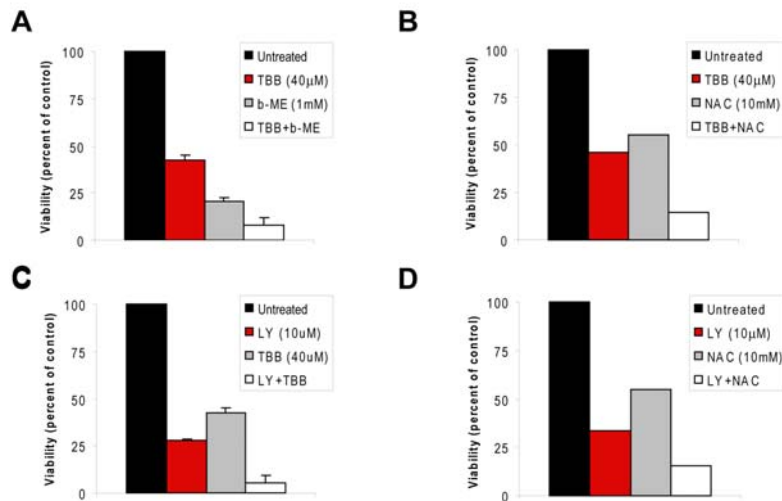


Figure S17. Cooperative effects of combinatorial treatment of HPB-ALL cells with pharmacological antagonists of PI3K, CK2 and ROS. (A,B) Inhibition of CK2 and ROS scavenging cooperate in inducing T-ALL cell death. HPB-ALL cells were cultured with the indicated concentrations of TBB and β -ME (A), TBB and NAC (B) or their combination and viability was assessed at 24h. (C-F) Inhibition of PI3K signaling cooperates with inhibition of CK2 and ROS scavenging in inducing T-ALL cell death. HPB-ALL cells were cultured with the indicated concentrations of LY294002 (LY) and TBB (C), LY and DRB (D), LY and β -ME (E), LY and NAC (F) or their combination and viability was assessed at 24h. Percent viability relative to untreated control (vehicle alone) is indicated for each condition (average \pm sem of duplicates).



E

Cooperative effect			
Combinations of Inhibitors of	Frequency	Median	Range
PI3K + CK2	5/6 (83%)	2.80	1.19 - 5.27
PI3K + ROS	3/6 (50%)	1.78	1.40 - 2.12
CK2 + ROS	5/6 (83%)	2.59	1.52 - 6.00

Figure S18. Cooperative effects of combinatorial treatment of primary T-ALL cells with pharmacological antagonists of PI3K, CK2 and ROS. (A,B) Inhibition of CK2 and ROS scavenging cooperate in inducing T-ALL cell death. Primary leukemia cells were cultured with the indicated concentrations of TBB and β -ME (A), TBB and NAC (B) or their combination and viability was assessed at 48h. (C-F) Inhibition of PI3K signaling cooperates with inhibition of CK2 and ROS scavenging in inducing T-ALL cell death. Primary leukemia cells were cultured with the indicated concentrations of LY294002 (LY) and TBB (C), LY and NAC (D) or their combination and viability was assessed at 48h. Percent viability relative to untreated control (vehicle alone) is indicated for each condition. Representative results out of six patients analyzed are shown. (E) Summary of results from all patients evaluated. Frequency refers to the percentage of primary samples for which cooperative effects were observed. Median and Range refer to the 'fold increase' in inhibitory effect of combination in relation the most effective single agent.

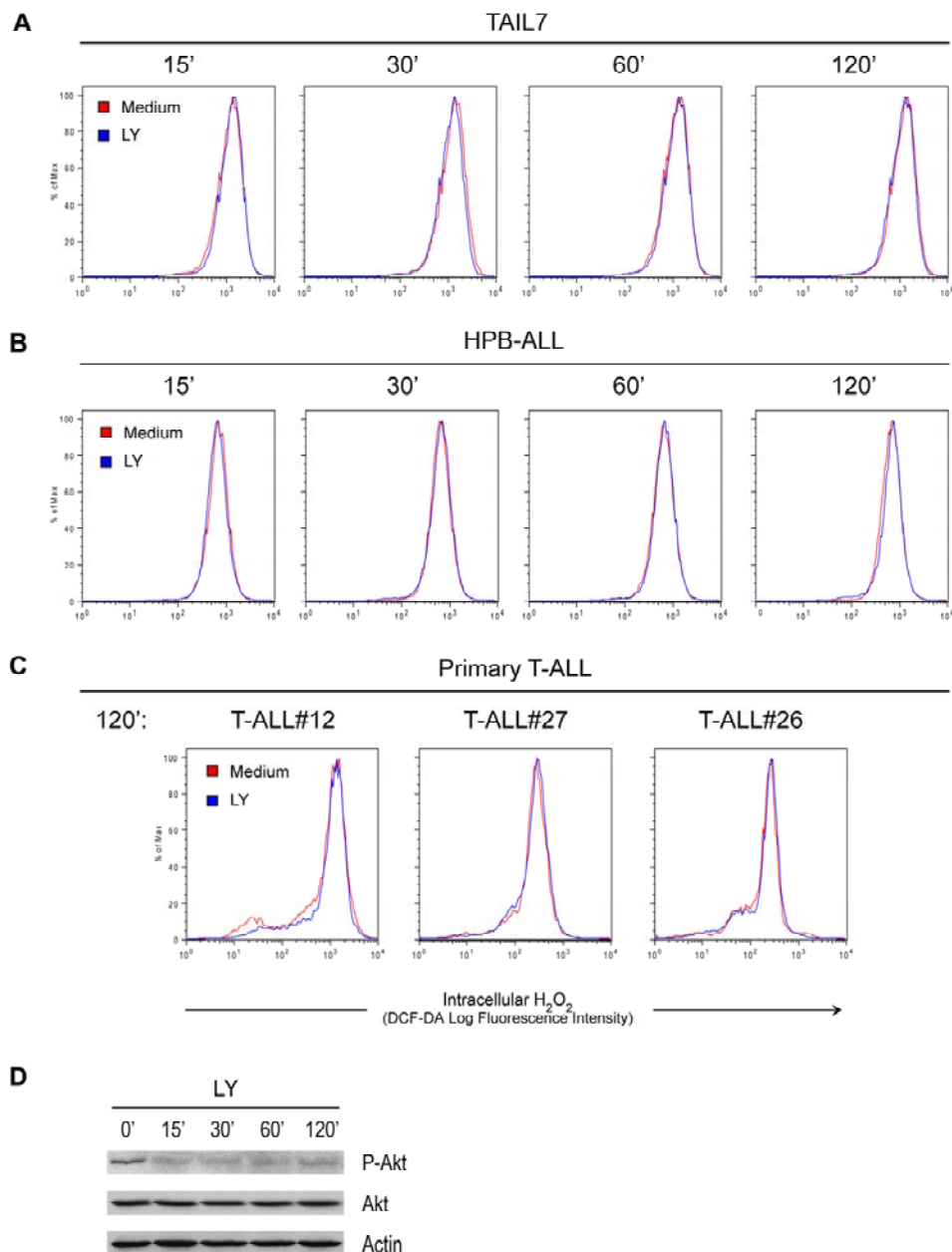
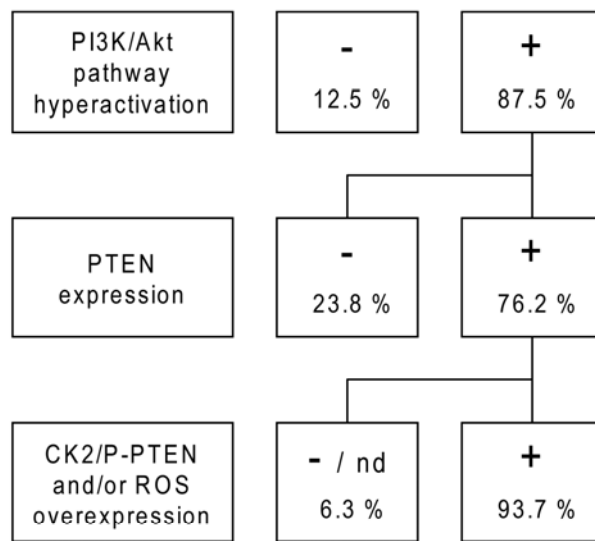


Figure S19. PI3K inhibition does not affect ROS levels in T-ALL cells. T-ALL cell lines TAIL7 (A) and HPB-ALL (B), and three different primary T-ALL patient samples (C) were treated with 25 μ M LY294002 (LY) for the indicated time points and intracellular H₂O₂ levels were analyzed by flow cytometry using the ROS-sensitive dye DCF-DA. (D) A proportion of HPB-ALL cells from the same experimental setting were lysed and phosphorylation of Akt (P-Akt) was determined by immunoblot to confirm that LY was effectively inhibiting PI3K. Cells lines were also treated with 50 μ M LY with similar results (not shown).

A**B**

CK2/P-PTEN	ROS	%
+	+	44
+	n.d.	19
+	-	19
n.d.	+	6
-	+	6
n.d.	n.d.	6
-	-	0

Figure S20. Percentage of PTEN-expressing PI3K/Akt pathway-‘hyperactivated’ primary T-ALL cases concomitantly overexpressing CK2/P-PTEN and/or ROS.

(A) Percentage of T-ALL specimens with PI3K/Akt pathway hyperactivation was calculated from a total of 24 cases. Percentage of PTEN-positive T-ALL cases was calculated within those that were classified as displaying PI3K/Akt pathway hyperactivation (n=21). In turn, percentage of cases with overexpression of CK2/P-PTEN and/or ROS was calculated within those that expressed PTEN (n=16). (B) Detailed distribution of overexpression of CK2/P-PTEN and/or ROS within PTEN-expressing PI3K/Akt pathway-‘hyperactivated’ primary T-ALL cases. + denotes high levels; - denotes normal levels; n.d. – not determined. Approximate percentages are indicated.

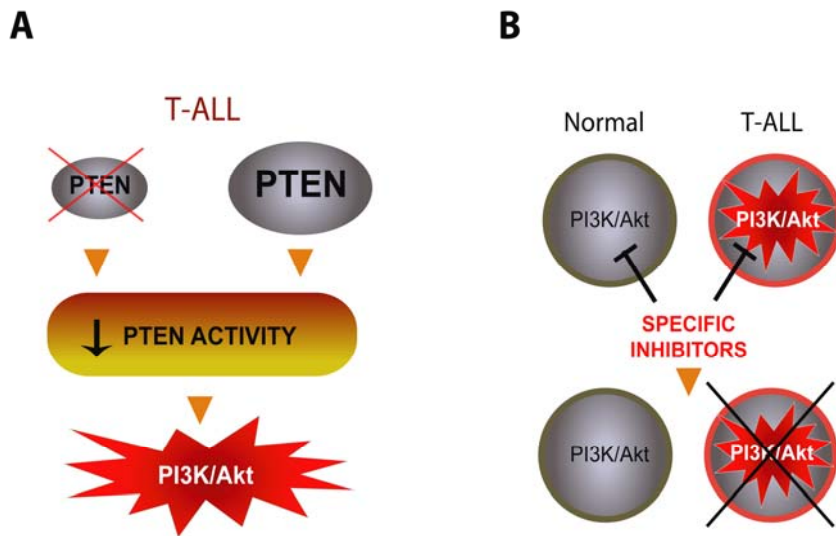


Figure S21. Summary of conclusions. (A) A minority of primary T-ALL cells lack PTEN protein expression as a consequence of PTEN gene alterations, whereas most specimens actually show increased PTEN expression that associates with PTEN phosphorylation and inactivation. Oxidation further promotes functional downregulation of PTEN activity. PTEN inactivation in leukemia cells contributes to constitutive hyperactivation of PI3K/Akt signaling pathway. (B) In contrast to their normal counterparts, T-ALL cells are clearly sensitive to inhibition of PI3K, upstream regulators and downstream effectors. Hence, specific small molecule inhibitors of PI3K/Akt signaling pathway may be valuable therapeutic tools for T-cell leukemia.

Supplemental Materials and Methods

Fluorescence-activated cell Sorting of thymic subpopulations. Normal thymocytes (10^8 cells) were surface stained with anti-CD3 APC-Alexa Fluor 750 (Ebioscience), anti-CD4 PE (Pharmingen), and anti-CD8 FITC (Pharmingen) conjugated antibodies at a 1:50 dilution. Cells were washed in PBS and CD8⁺CD3⁺ (SP8), CD8⁺CD4⁺CD3⁺ (TP), CD3⁺ and CD3⁻ thymocyte subpopulations were isolated using a FACSAria cell sorter (Becton-Dickinson). Sorted populations with purities ranging from 83% to 99% were immediately lysed or used for culture experiments.

PTEN intracellular staining. Cells were fixed in 1% paraformaldehyde, permeabilized in 0.05% saponin and incubated 30 minutes at 4°C with a mouse anti-PTEN monoclonal antibody (Santa Cruz Biotechnology) at 1:100 dilution. Primary antibody was detected with Alexa488-conjugated donkey anti-mouse antibody (Molecular Probes). Thymocyte samples were surface stained with anti-CD4PE and anti-CD8 APC (Ebioscience) conjugated antibodies. Samples were analyzed by flow cytometry to quantify mean fluorescence intensity using FACSCanto flow cytometer (Becton-Dickinson) and FlowJo (Tree Star) software.

PIP3 levels in surface-stained cells. Cells were washed in PBS and fixed in 1% paraformaldehyde, blocked with 10% normal goat serum in PBS, and incubated overnight at 4°C with mouse anti-PIP3 monoclonal antibody (Echelon) at 1:10 dilution. Primary antibody was detected with Alexa488-conjugated donkey anti-mouse antibody (Molecular Probes). Samples were washed and surface stained with anti-CD3 APC-Alexa Fluor 750, anti-CD4PE and anti-CD8 APC antibodies. Samples were acquired on a FACSCanto flow cytometer and analyzed with FlowJo software.

Intracellular ROS levels in surface-stained cells. Cells were stained with DCF-DA as described previously, washed and surface stained with anti-CD3 APC-Alexa Fluor 750, anti-CD4 PE-Cy7 (Ebioscience), and anti-CD8 APC antibodies. Samples were acquired on a FACSCanto flow cytometer and analyzed with FlowJo software.