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ORIGINAL ARTICLE

Casein kinase 2 controls the survival of normal thymic and leukemic γδ T cells via promotion of AKT signaling

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The thymus is the major site for normal and leukemic T-cell development. The dissection of the molecular determinants of T-cell survival and differentiation is paramount for the manipulation of healthy or transformed T cells in cancer (immuno)therapy. Casein kinase 2 (CK2) is a serine/threonine protein kinase whose anti-apoptotic functions have been described in various hematological and solid tumors. Here we disclose an unanticipated role of CK2 in healthy human thymocytes that is selective to the γδ T-cell lineage. γδ thymocytes display higher (and T-cell receptor inducible) CK2 activity than their αβ counterparts, and are strikingly sensitive to death upon CK2 inhibition. Mechanistically, we show that CK2 regulates the pro-survival AKT signaling pathway in γδ thymocytes and, importantly, also in γδ T-cell acute lymphoblastic leukemia (T-ALL) cells. When compared with healthy thymocytes or leukemic αβ T cells, γδ T-ALL cells show upregulated CK2 activity, potentiated by CD27 costimulation, and enhanced apoptosis upon CK2 blockade using the chemical inhibitor CX-4945. Critically, this results in inhibition of tumor growth in a xenograft model of human γδ T-ALL. These data identify CK2 as a novel survival determinant of both healthy and leukemic γδ T cells, and may thus greatly impact their therapeutic manipulation.

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INTRODUCTION

T cells develop in the thymus. The dissection of the cell-intrinsic and -extrinsic signals that regulate thymocyte survival, proliferation and differentiation is critical to understand their potential for transformation and to devise new therapies for T-cell acute lymphoblastic leukemia (T-ALL).

T-cell commitment is coupled to somatic T-cell receptor (TCR) rearrangements, generating thymocytes bearing either an αβ or a γδ TCR.[1] The expression of a pre-TCR composed of TCRβ and the invariant pTα chain in αβ thymocyte progenitors results in a massive proliferative burst ('β-selection') that dictates that αβ T cells largely outnumber their γδ counterparts. Likely a consequence, although significant progress has been made in our understanding of human αβ T-cell development, the molecular determinants of γδ thymocytes remain poorly characterized.[1]

Most of what we know about thymic γδ T-cell differentiation comes from studies performed in mice, showing how various receptors (namely, TCRγδ, CD27 and LTβR) and downstream transcription factors (such as Id3, Sox13, TCF1 and Lef1) control various maturation steps, from divergence from the αβ lineage to the acquisition of effector functions such as pro-inflammatory cytokine production.[2–7] In contrast, much less is known about human thymic γδ T-cell differentiation. Notwithstanding, we recently showed that interleukin (IL-2) or IL-15 differentiate human γδ thymocytes into cytotoxic type 1 effector T cells, rendering them highly efficacious against leukemic cells *in vitro* and *in vivo*.[8,9]

γδ thymocytes can themselves transform into leukemic cells, causing a rare ($<10\%$ of all cases) form of T-ALL with distinctive clinical features.[10–12] Given that malignant γδ T cells have been very poorly studied and lack defined molecular targets, we have here addressed the potential role of casein kinase 2 (CK2), a signaling effector molecule previously implicated in chronic lymphocytic leukemia,[13,14] multiple myeloma,[15] B-ALL,[16,17] T-ALL[18,19] and other hematological disorders (reviewed in Piazza *et al.*[20]).

CK2 is a ubiquitous and constitutively activated serine/threonine protein kinase that regulates multiple pathways including phosphatidylinositol 3-kinase/AKT and WNT signaling, nuclear factor-κB transcription and the DNA damage response.[20] CK2 displays pro-survival and anti-apoptotic functions that were described in several cancer cell types. CK2 is frequently overexpressed or hyperactivated in both solid tumors and hematological malignancies, thus making it a promising target for cancer treatment.[20] In contrast, the physiological function of CK2 in nontransformed cells is less established. Recent studies in mice have demonstrated that CK2 activity is necessary for peripheral T-cell activation and function: interference with CK2 signaling impaired $CD4^+$ T-cell activation and differentiation into T helper type 2 (Th2) or Th17 cells,[21], whereas the genetic deletion of CK2 in $CD4^+$ $Foxp3^+$ regulatory T cells abolished their suppressive activity against allergy-promoting Th2 cells.[22] However, no functional role has yet been attributed to CK2 in the human thymus.

In this study we identified a novel role for CK2 in controlling the survival of normal γδ thymocytes and γδ T-ALL cells. We analyzed CK2 activity in γδ versus αβ thymocytes and T-ALL cells, its regulation by cell-extrinsic signals, the downstream signaling mechanisms and the effect of its inhibition *in vitro* and *in vivo* in a xenograft model of γδ T-ALL.

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MATERIALS AND METHODS

Statement of Ethics

Thymic specimens (from newborn to 15-year-old children) were obtained during pediatric corrective cardiac surgery after parents' written informed consent. The study was approved by the Ethics Board of Faculdade de Medicina da Universidade de Lisboa. Primary T-ALL blasts derived from diagnostic samples (peripheral blood or bone marrow), obtained after informed content and amplified upon xenografting into NSG (NOD.Cg-Prkdc(scid)Il2rg(tm1Wjll)/SzJ) mice.

Isolation, cell culture and viral transduction

Thymic T cells were collected after thymus tissue dispersion and separation by Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA) density gradient separation. TCRγδ-positive T cells were isolated (to >97% purity) by magnetic positive selection; TCRαβ-positive T cells were isolated (to >96% purity) by magnetic positive selection from the TCRγδ-negative fraction (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were used as fresh or, when indicted, cells were cultured at 37 °C with 5% CO_2 in complete RPMI-1640 as previously described[23] on indicated conditions. For long-term *in vitro* cell culture of thymocytes (7 days), recombinant human IL-2 was added to the medium. The PEER T-ALL (DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and MOLT-4T-ALL (ATCC CRL-1582) were cultured in 90% RPMI-1640+10% fetal bovine serum following the manufacturer's instructions. When indicated, PEER cell line was transduced using a bicistronic retroviral DNA construct, either empty vector (LZRS) expressing only IRES followed by eGFP (LZRS-IRES-eGFP) or vector co-expressing myrPKB/AKT (constitutively activated AKT) and eGFP (LZRS-myrPKB/AKT-IRES-eGFP) as previously described.[24] To increase the percentage of transduced cells for the following experiments, GFP^+ cells were sorted (≈100% purity) using a FACSAria high-speed cell sorter (BD Biosciences, San Jose, CA, USA).

Chemicals and antibodies

Anti-human monoclonal antibodies were used against: CD3 (UCHT1), CD27 (LG.7F9), CD4 (RPA-T4), CD7 (4H9) and panαβTCR (IP26) from eBioscience (San Diego, CA, USA); CD28 (CD28.2), CD8 (SK1), CD45 (HI30), Vδ2 (B6), CD3 (OKT3), CD45RA (HI100), Annexin-V and 7-aminoactinomycin D (7-AAD) from Biolegend (San Diego, CA, USA); panγδTCR (5A6.E9) from ThermoFisher (Rockford, IL, USA); Vδ1 (REA173) from Miltenyi Biotech; p-S129-AKT, AKT, p-S9-GSK3β, GSK3β, p-S380-PTEN, PTEN, p-S235/236-S6 and S6 from Cell Signaling (Danvers, MA, USA); Calnexin and GAPDH from Sicgen (Cantanhede, Portugal); 7-AAD from Invitrogen (Carlsbad, CA, USA) and B-cell lymphoma 2 (Bcl-2) from Dako (Glostrup, Denmark). Recombinant human sCD27 ligand and recombinant human IL-2 were purchased from Peprotech (Rocky Hill, NJ, USA); CX-4945 (Silmitasertib) from Adooq Bioscience (Irvine, CA, USA) and Biorbyt (Cambridge, UK); TBB from Sigma-Aldrich; TG-003 and Harmine from Focus Biomolecules (Plymouth Meeting, PA, USA).

Flow cytometry, cell viability, cell cycle and proliferation analysis

Cells were stained for the indicated cell surface markers, and intracellular staining was performed using fixation/permeabilization and permeabilization buffers (both from eBioscience), following the manufacturer's instructions. Cell apoptosis was analyzed by flow cytometry using Annexin-V/7-AAD staining as previously reported.[25] For cell cycle analysis, cells were stained for 1 h at 37 °C with 30 μl of 7-AAD (BD Pharmingen, San Diego, CA, USA) in permeabilization buffer (eBioscience). CFSE-based proliferation assays (CellTrace CFSE kit, Invitrogen, final concentration 0.5 mM) were performed as previously described.[4] Samples were acquired using LSRFortessa or Accuri C6 (both from BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

CK2 kinase activity assay

CK2 activity was measured in cell lysates (from equal cell numbers) using the casein kinase-2 assay kit (17-132) from Upstate Biotechnology (Lake Placid, NY, USA), following the manufacturer's instructions. Briefly, total protein lysates were incubated for 10 min at 30 °C in a reaction mixture containing: CK2α-specific peptide, [γ-32 P]ATP and protein kinase A inhibitor cocktail. The radioactivity incorporated into the substrate was determined in P81 phosphocellulose paper-squares by scintillation counting as previously reported.[14] CK2 activity in $γδ^+$ and $αβ^+$ xenograft-derived blasts was measured on samples that showed comparable percentages of human engraftment (defined by fluorescence-activated cell sorting analysis based on the expression of CD45 and CD7 antigens).

Western blot analysis

Cell lysates were used for immunobloting as previously described.[14] Briefly, the cells were lysed, at 4 °C, in cold lysis buffer (50 mM Tris (pH 7.6), 150 mM EDTA, 1% Nonidet P-40 in phosphate-buffered saline) enriched with protease and phosphatase inhibitor cocktails (Roche, Burgess Hill, UK). The total proteins were quantified using a Bradford assay (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. Equal amounts of total protein was denatured in Laemmli buffer (Bio-Rad), boiled for 5 min at 95 °C and loaded in a 10% SDS–polyacrylamide gel electrophoresis. After electrophoretic separation, the proteins were transferred to nitrocellulose blotting paper (Amersham Biosciences, Little Chalfont, UK). The membranes were blocked with 5% bovine serum albumin and 0.5% Tween-20 (Sigma-Aldrich) in phosphate-buffered saline and probed with the indicated primary antibodies overnight. After rinse, the membranes were probed using appropriate horseradish peroxidase-conjugated secondary antibodies and developed by chemiluminescence using the ChemiDoc XRS + imaging system (Bio-Rad).

In vivo mouse experiments

All experimental procedures were performed in strict accordance with the recommendations of the European Commission (Directive 2010/63/UE), French National Committee (87/848) and Portuguese authorities (Decreto-Lei 113/2013) for the care and use of laboratory animals. TCRαβ- or TCRγδ-positive T-ALL cells obtained from patient diagnostic were injected into 8–12-week-old NSG mice (1×10^6 cells/mouse, tail vein injections). Mice were monitored weekly by flow cytometry for human leukemic load ($hCD7^+$, $hCD45^+$ cells) in peripheral blood. Mice were killed when terminally ill and blast cells from bone marrow were collected. For the *in vivo* experiment of γδ T-ALL treatment, 10–12-week-old NRGS mice were injected subcutaneously in the right flank with 2×10^6 PEER cells resuspended in 100 μl of phosphate-buffered saline. At day 20, all mice presented palpable tumors (100–150 mm^3) and were randomly distributed into two groups ($n \geqslant 3$). The animals were treated with CX-4945 (75 mg/kg), by oral gavage twice daily (b.i.d.) or vehicle control (25 mM sodium bisphosphate buffer) as previously described.[26] Mice were monitored daily and weighed frequently. Tumors were measured every 2 days with a caliper and tumor volume was calculated (volume = (length × width)^2/2). At day 13, after starting the treatment, all mice were killed (an ethical requirement for the control group, when tumor reached 2000 mm^3).

Statistical analysis

Statistical significance of differences between indicated conditions was assessed using Student's *t*-test with Welch's correction and is indicated when significant as $*P < 0.05$; $**P < 0.01$; $**P < 0.001$. All statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA).

RESULTS

Human γδ thymocytes have enhanced CK2 activity and are highly sensitive to its inhibition

This study initiated with the analysis of CK2 activity in normal thymocyte subsets obtained from pediatric thymic biopsies. We measured CK2 activity using a substrate-specific kinase assay in freshly isolated TCRγδ+ or TCRαβ+ cells, and unexpectedly found twofold higher activity in γδ thymocytes relative to their αβ counterparts (Figure 1a). To address its physiological relevance, we treated thymocytes for 24 h with a highly specific ATP-competitive inhibitor of CK2, CX-4945.[27] Flow cytometry analysis of Annexin-V/7-AAD-stained cells revealed increased apoptosis of γδ compared with $CD4^+$ and $CD8^+$ αβ thymocytes (Figure 1b). To assess the longer-term impact of CX-4945 treatment, thymocytes were cultured in the presence of TCR plus costimulation for 7 days. The γδ thymocytes were highly susceptible to apoptosis upon CK2 inhibition in a dose dependent-manner (Figure 1c). In contrast, as previously reported,[18] $CD4^+$ or $CD8^+$ αβ thymocyte survival was not significantly affected following CK2 inhibition (Figure 1c).

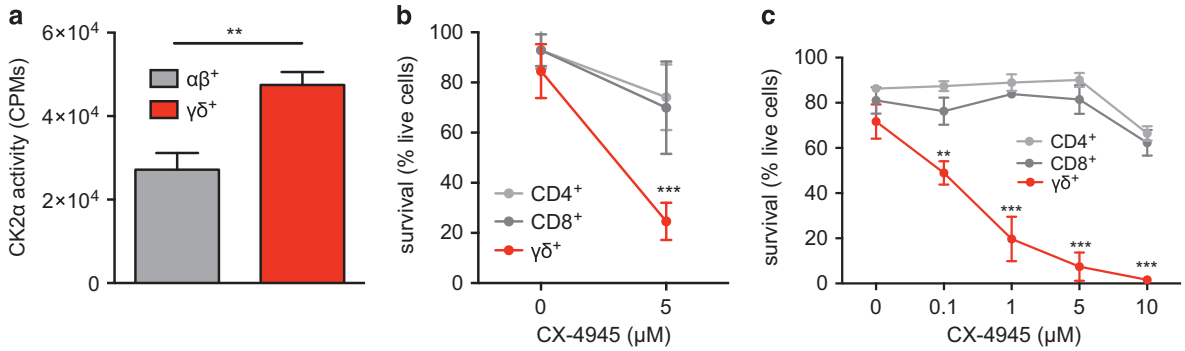

Figure 1. Human γδ thymocytes have enhanced CK2 activity and are highly sensitive to CX-4945. (**a**) *In vitro* CK2α activity (kinase assay) in freshly isolated human thymic γδ and αβ T-cells (2×10^6 cells per assay). CPM, counts per min. (**b**) Survival (% of live cells) of human thymic γδ, $CD4^+$ and $CD8^+$ T cells following 24 h of incubation with 5 μM of the CK2 inhibitor, CX-4945, analyzed by flow cytometry using Annexin-V/7-AAD staining. (**c**) Survival (% of live cells) of human thymic γδ, $CD4^+$ and $CD8^+$ T cells to different concentrations of CX-4945 (or vehicle), analyzed by Annexin-V/7-AAD staining following 7 days in culture with recombinant human IL-2 (rhIL-2) plus CD3+CD27 or CD3+CD28 stimulation of sorted thymic γδ or αβ T cells, respectively. Data in this figure are representative of at least three independent experiments; $^{**}P < 0.01$, $^{***}P < 0.001$ (*T*-test).

These data revealed that healthy γδ thymocytes are exquisitely dependent on their high basal CK2 activity for survival, and thus extremely sensitive to chemical inhibition using CX-4945. To verify that these effects were mediated by inhibition of CK2 rather than other kinases, CLK2 and DYRK1A, recently reported to be sensitive to CX-4945 treatment,[28,29] we also tested another CK2 inhibitor, 4,5,6,7-tetrabromobenzotriazole (TBB); TG-003 that specifically targets CLK2;[28,30] and Harmine that selectively inhibits DYRK1A.[31] Importantly, only the other CK2 inhibitor, TBB, reproduced the effects of CX-4945, whereas TG-003 and Harmine failed to affect γδ thymocyte survival (Supplementary Figure S1). These results strongly suggest that the physiological target of CX-4945 in γδ thymocytes is CK2.

CK2 activity in γδ thymocytes is modulated by TCR stimulation and promotes AKT signaling

We next asked which signals regulated CK2 activity in γδ thymocytes. Very few studies have documented CK2 modulation by physiological stimuli in T cells.[21,32] When we stimulated (for 6 h) isolated thymocyte subsets via the TCR complex using agonist anti-CD3ε antibodies, we observed an approximately threefold enhancement of CK2 activity selectively in γδ thymocytes (Figure 2a). We also tested the impact of costimulation, particularly through CD27 that we have shown to play a major role in γδ T-cell development and expansion.[4,33,34] However, the addition of soluble recombinant CD27-ligand/CD70 (sCD70) had no additive effect on CK2 activity (Figure 2a). Thus, our data suggest that CK2 activity in healthy γδ thymocytes is modulated primarily by TCR signals.

To gain insight into the downstream effects of CK2 signaling and its inhibition in γδ thymocytes, we focused on the AKT signaling pathway that is involved in cell survival and proliferation and known to be regulated by CK2 in both normal and malignant αβ T lymphocytes.[18,35] We observed that TCR/CD27 stimulation inhibited PTEN (phosphatase and tensin homolog), as measured by the increase in its phosphorylated form, and potentiated the AKT signaling pathway in γδ but not αβ thymocytes, as shown by the phosphorylation of AKT and its downstream targets glycogen synthase kinase-3β (GSK3β) and S6 (Figure 2b and Supplementary Table S1). These effects were completely reversed by CX-4945 (Figure 2b). As functional outcomes of CK2 inhibition, we observed decreased γδ thymocyte proliferation (Figure 2c) and survival (Figure 2d). Moreover, in agreement with the implication of AKT signaling downstream of CK2, we found a similarly striking effect on γδ thymocyte survival upon treatment with the specific AKT inhibitor, MK-2206 [ref. 36] (Figure 2d).

CD27-dependent upregulation of CK2 activity and downstream AKT signaling in γδ T-ALL

We next asked how CK2 activity would affect γδ T-ALL. First, we compared CK2 activity in normal γδ and αβ thymocytes versus γδ and αβ T-cell blasts obtained from T-ALL patients (and expanded in NSG mice, with similar engraftment, as detailed in the Materials and methods section). As expected, αβ T-ALL cells displayed higher levels of CK2 activity than αβ thymocytes (Figure 3). Notably, we detected markedly higher CK2 activity in γδ T-ALL cells as compared with healthy thymocytes and αβ T-ALL blasts (Figure 3). Moreover, the γδ T-ALL cell line PEER reproduced the very high CK2 activity observed in γδ T-ALL blasts (Figure 3), making it a good model for further biochemical and functional CK2 tests in γδ T-ALL.

The CK2 inhibitor, CX-4945, suppressed CK2 activity in γδ T-ALL cells in a dose-dependent manner (Figure 4a). As with healthy γδ thymocytes, the effects of CX-4945 were only reproduced by another CK2 inhibitor, TBB, but not by TG-003 or Harmine that selectively target CLK2 and DYRK1A, respectively (Supplementary Figure S1). Thus, in γδ T-ALL cells also, CX-4945 exerts its effects by suppressing CK2 activity. On the other hand, CK2 activity was enhanced upon activation, with CD27 costimulation having a synergistic contribution in γδ T-ALL cells (Figure 4b), in contrast with γδ thymocytes (Figure 2a). Of note, PEER cells are $CD27^+$ $V\delta1^+$ γδ T-ALL cells (Supplementary Figure S2). A CD27-dependent effect was also observed on AKT signaling (Figure 4c), and was completely abrogated upon CX-4945 treatment, also in a dose-dependent manner (Supplementary Figure S3).

Functionally, CK2 inhibition led to γδ T-ALL cell cycle arrest at G2/M phase, cell apoptosis and decreased Bcl-2 protein levels (Figure 4d). To further examine the functional impact of AKT activation downstream from CK2, we tried to rescue this apoptotic phenotype by expressing a myristoylated, constitutively active form of AKT.[37] Cells expressing myristoylated AKT displayed high levels of AKT phosphorylation that were insensitive to CK2 inhibition (Figure 4e). Importantly, AKT hyperactivation partially rescued γδ T-ALL survival under CX-4945 treatment (Figure 4f). On the other hand, CK2 activity was not affected by AKT hyperactivation (Supplementary Figure S4), collectively suggesting that AKT phosphorylation is

downstream rather than upstream of CK2, and that AKT is a key mediator of CK2 functions.

γδ T-ALL cells are highly sensitive to CK2 inhibition *in vitro* and *in vivo*

To further evaluate the functional impact of CK2 inhibition on γδ T-ALL survival, we compared the effect of CX-4945 treatment on primary γδ versus αβ T-ALL cells, as well as representative cell lines, PEER and MOLT-4, respectively.

Upon 48 h of *in vitro* treatment with CX-4945, we observed increased cell apoptosis in primary γδ T-ALL samples compared with αβ T-ALL cells (Figure 5a). We performed a more detailed test with the T-ALL cell lines, at various time points of incubation with CX-4945, and found a profound and dose-dependent effect on PEER (γδ T-ALL) that were significantly more susceptible to apoptosis that MOLT-4 (αβ T-ALL) cells (Figure 5b). These data suggest that γδ T-ALL cells, like healthy γδ thymocytes (Figures 1b and c), are considerably more sensitive to CK2 inhibition than αβ T-ALL cells.

Finally, the increased sensitivity of γδ T-ALL cells to CX-4945 treatment *in vitro* led us to explore its therapeutic potential *in vivo*. For this purpose, we established a xenograft model of human γδ T-ALL by injecting 2×10^6 PEER cells subcutaneously in immune-deficient NRGS (NOD-Rag1^{null} IL2Rgamma^{null}) mice. After the detection of palpable tumor, mice were equally distributed

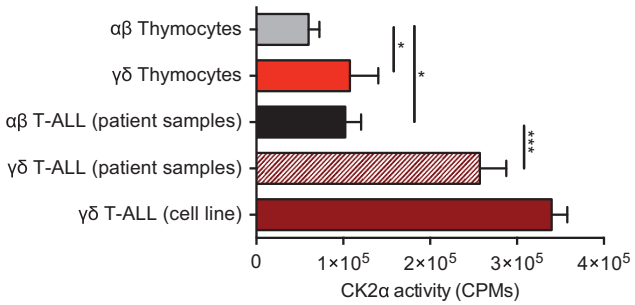

Figure 3. γδ T-ALL cells display higher CK2 activity than αβ counterparts. *In vitro* CK2α activity (kinase assay; 6.6×10^6 cells per assay) in freshly isolated γδ ($n=4$) and αβ ($n=4$) thymocyte samples; γδ ($n=6$) and αβ ($n=14$) T-cell samples obtained from T-ALL patients and expanded in NSG mice (as described in the Materials and methods); and the γδ T-ALL cell line, PEER ($n=4$). *T*-test, $^*P<0.05$, $^{***}P<0.001$.

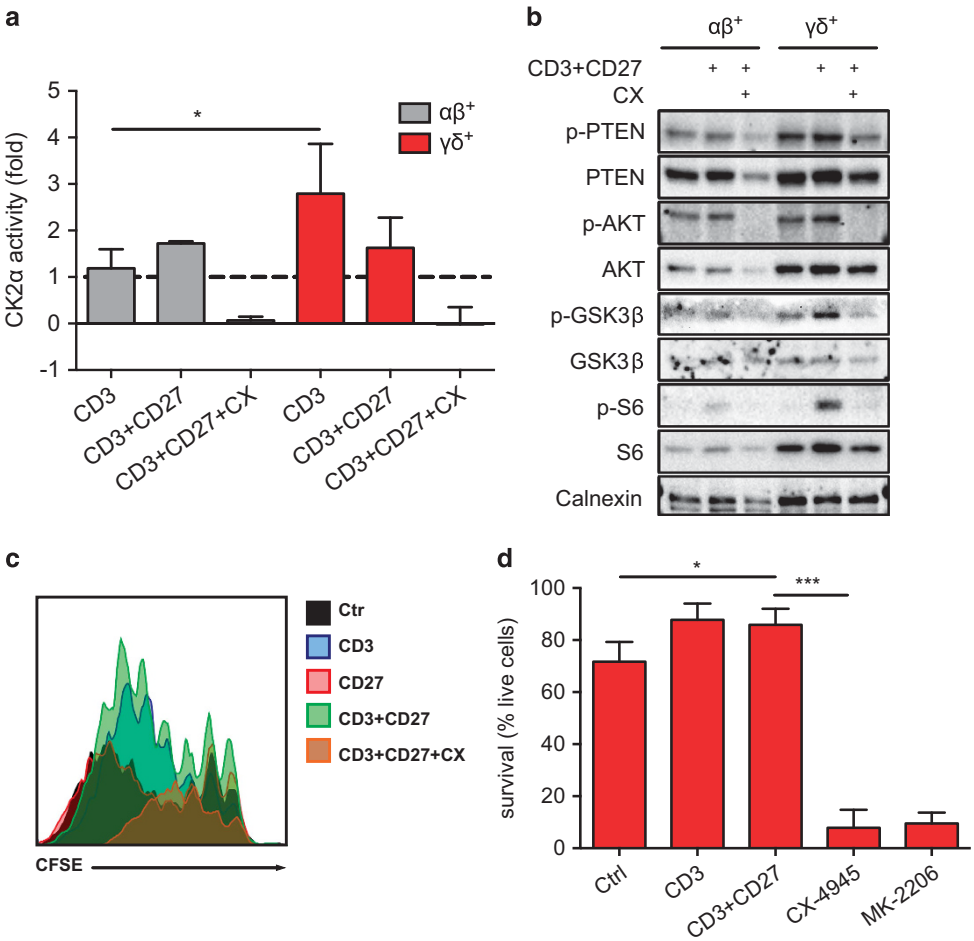

Figure 2. CK2 activity in γδ thymocytes is modulated by TCR stimulation and activates AKT signaling. (**a**) *In vitro* CK2α activity in sorted γδ and αβ thymocytes (2×10^6 cells per sample) after 6 h of stimulation with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27) or plus 5 μM CX-4945 (CD3+CD27+CX); values were normalized to unstimulated control (dashed line). (**b**) Western blot analysis of (phospho)proteins implicated in AKT signaling, in γδ and αβ thymocytes (1×10^6 cells per sample) stimulated as in (**a**). (**c**) Proliferation (CFSE dilution assay) of γδ thymocytes after 7 days in culture with recombinant human IL-2 (rhIL-2) under the indicated conditions: medium only (Ctrl); anti-CD3 antibody stimulation (CD3); soluble CD27-ligand (CD27); their combination (CD3+CD27); and with 5 μM CX-4945 (CD3+CD27+CX). (**d**) Survival (% of live cells) of γδ thymocytes after 7 days of stimulation (or not, Ctrl for control) with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27), plus 5 μM of CX-4945 or 10 μM of MK-2206. Data in this figure are representative of at least three independent experiments; $^*P<0.05$, $^{***}P<0.001$ (*T*-test).

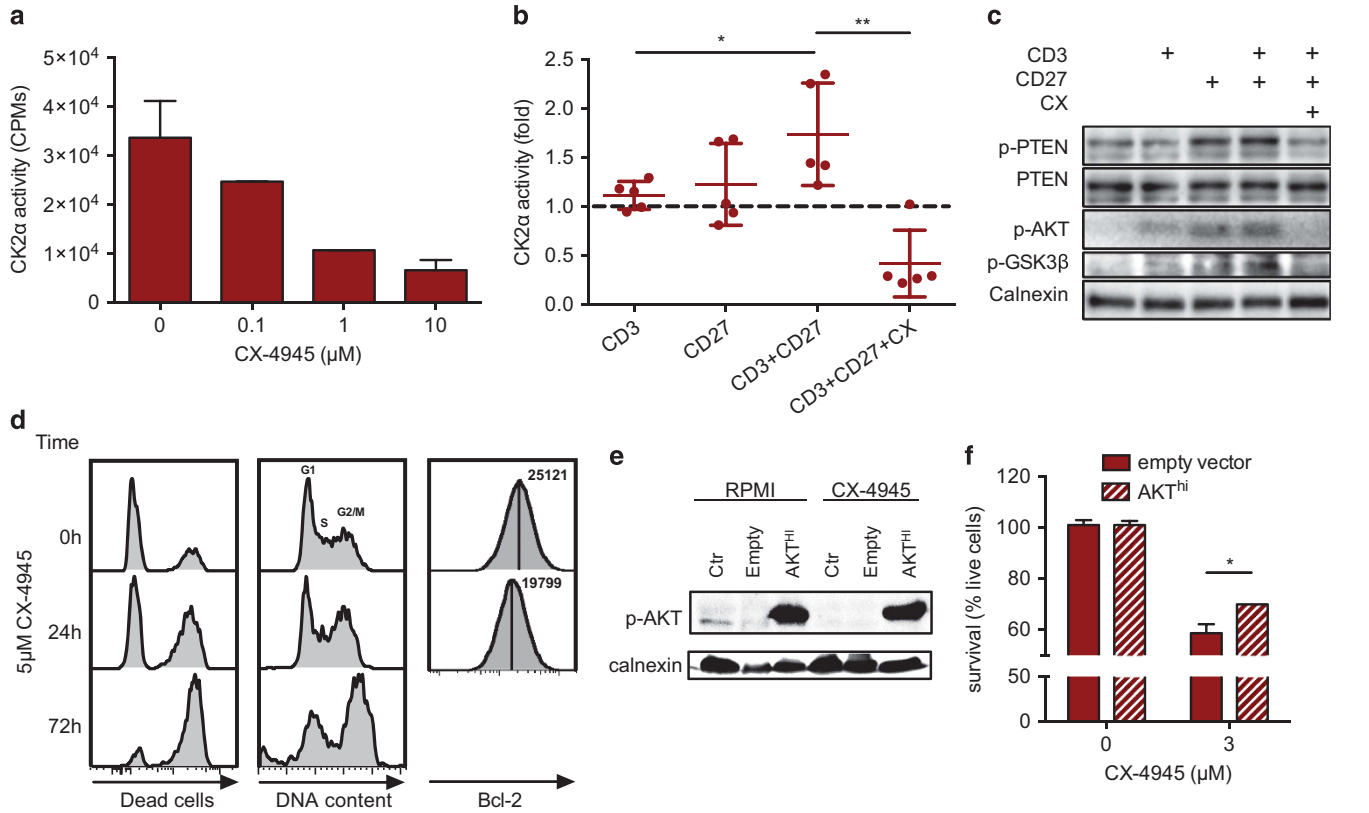

Figure 4. CK2 activity in γδ T-ALL cells is potentiated by CD27 costimulation and promotes AKT signaling. (**a**) CK2α activity in the γδ T-ALL cell line, PEER (2×10^6 cells per condition), after 6 h of treatment with indicated concentrations of CX-4945. (**b**) CK2α activity in lysates from γδ T-ALL (PEER) cells (2×10^6 cells per condition) after 6 h of stimulation under the indicated conditions (*T*-test, $^*P<0.05$; $^{**}P<0.01$). (**c**) Western blot analysis of (phospho)proteins implicated in AKT signaling, in γδ T-ALL (PEER) cells treated for 6 h with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27) or plus 5 μM CX-4945 (CD3+CD27+CX). Data are representative of five independent experiments. (**d**) Flow cytometry analysis of apoptosis (Annexin-V$^+$; left panel), cell cycle/DNA staining (middle panel) and intracellular Bcl-2 protein staining (right panel; values indicate mean fluorescence intensity (MFI)) of γδ T-ALL (PEER) cells treated with CX-4945 (5 μM) during the indicated times. (**e**, **f**) Western blot analysis of phospho-AKT (and calnexin loading control) (**e**) and cell survival after 48 h (**f**) of PEER cells transduced with a bicistronic retroviral DNA construct: either empty vector (LZRS) expressing only IRES followed by eGFP (LZRS-IRES-eGFP) or vector co-expressing myrPKB/AKT and eGFP (LZRS-myrPKB/AKT-IRES-eGFP) (AKT^{hi}) and treated with 3 μM CX-4945 or vehicle (*T*-test, $^*P<0.05$).

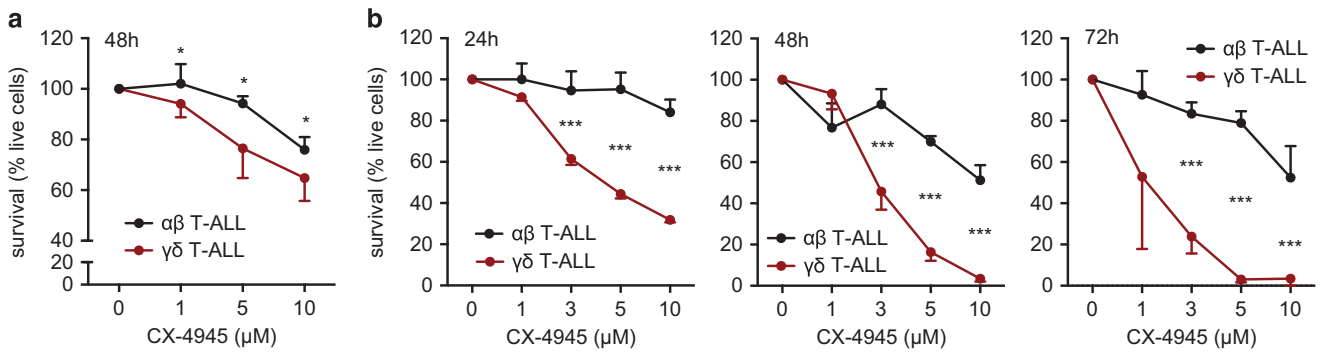

Figure 5. γδ T-ALL cells are more susceptible than αβ T-ALL to apoptosis induced by CX-4945. Flow cytometry analysis of the survival (Annexin-V/7-AAD staining) of (**a**) γδ ($n=5$) and αβ ($n=5$) T-cell blast samples (obtained from T-ALL patients and expanded in NSG mice) or (**b**) γδ (PEER) or αβ (MOLT-4) T-ALL cell lines, cultured for the indicated times with increasing concentrations of CX-4945 (*T*-test, $^*P<0.05$; $^{***}P<0.001$).

according to tumor burden into two groups to receive CX-4945 (orally, twice a day) or vehicle control. We observed a striking impact of CX-4945 treatment on tumor growth (Figure 6a). Upon killing the mice at day 18 (an ethical requirement for the control group), we scored great reductions in the CX-4945-treated group concerning the tumor weight (Figure 6b), as well its dissemination to the blood, bone marrow and spleen (Figures 6c–e). Of note, this therapeutic effect was dose dependent, as it was only observed with 75 mg/kg (Figures 6a–e) but not with 25 mg/kg (data not shown) of CX-4945. These data collectively demonstrate the potential of CK2 inhibition for treatment of γδ T-ALL.

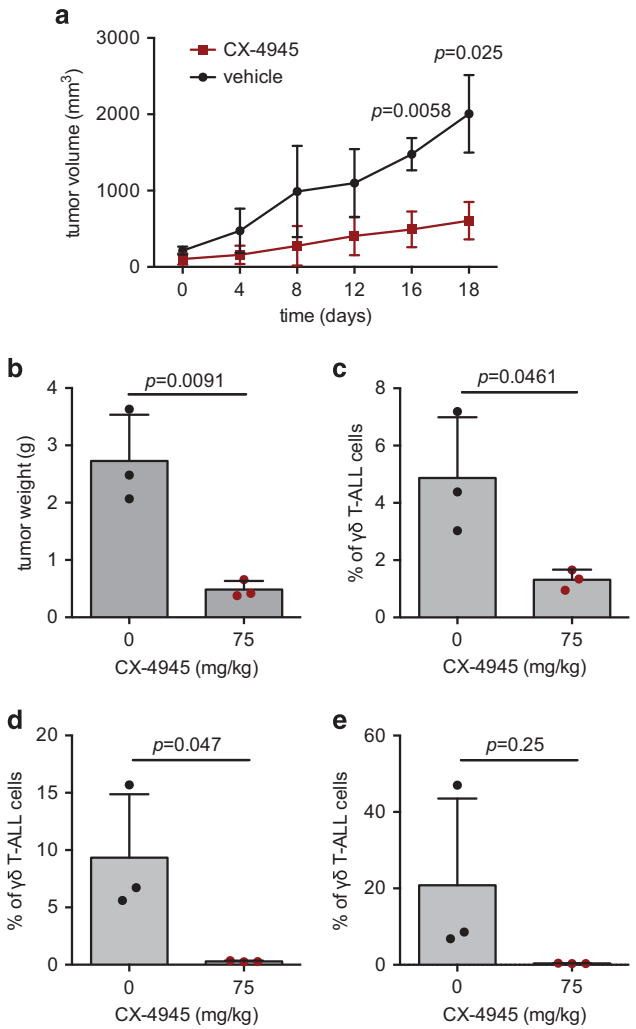

Figure 6. CX-4945 treatment inhibits γδ T-ALL growth *in vivo*. (**a**) Tumor volume following injection of 2×10^6 PEER γδ T-ALL cells subcutaneously into NRGS mice, treated with 75 mg/kg CX-4945 or vehicle (*T*-test, $^*P < 0.05$, $^{**}P < 0.01$). Day 0 refers to the start of treatment of mice bearing palpable tumors. (**b–e**) Tumor weight (**b**) or percentage of $CD45^+$ $CD7^+$ γδ T-ALL cells in the blood (**c**), bone marrow (**d**) or spleen (**e**) of mice killed after 18 days of treatment. Each dot represents an animal; *T*-test *P*-values are indicated.

DISCUSSION

Increased CK2 activity is typically associated with cell transformation in several hematological and solid tumors,[38] but few reports have demonstrated its relevance in physiological conditions.[32,39] Recent studies in mice have shown that epithelial cells and peripheral T cells depend on CK2 for their survival and function. CK2 was required for the survival of intestinal epithelial cells in inflammatory colitis,[40] for $CD4^+$ T-cell activation and differentiation into Th2 or Th17 cells[21] and for the suppressive function of $CD4^+$ $Foxp3^+$ regulatory T cells against allergy-promoting Th2 cells.[22] However, no physiological role has yet been attributed to CK2 in the human thymus or on healthy human T cells.

Here we identify a major role for CK2 that is restricted to the γδ lineage of human thymocytes. These display approximately twofold higher CK2 activity and are strikingly more sensitive to CK2 inhibition than their αβ counterparts. As we show that TCR stimulation increases (~2.5-fold) CK2 activity in γδ thymocytes, we may speculate that the different basal levels of CK2 activity in γδ versus αβ thymocytes are because of stronger TCR signals received during their development. It is well established that strong TCR signaling favors γδ over αβ T-cell lineage commitment[1,41–43] and further affects subsequent γδ thymocyte development.[44] In this line of reasoning, the high CK2 activity in agonist-selected γδ thymocytes could be an important pro-survival mechanism to counteract the activation-induced cell death underlying thymocyte negative selection.

We previously showed that αβ primary T-ALL cells display higher levels of CK2 expression and activity than immunophenotypically equivalent normal αβ thymocytes.[18] Most interestingly, we now demonstrate that the differential CK2 activity between the γδ and αβ T-cell lineages extends from healthy thymocytes to transformed T-ALL cells. Thus, primary γδ T-ALL cells displayed more than twofold higher CK2 activity compared with αβ T-ALL counterparts. As we previously showed that endogenous CK2 activity correlates with increased susceptibility to apoptosis upon CK2 inhibition,[14,18,19] this differential activity likely explains the higher sensitivity of γδ T-ALL cells observed in the present study.

Although rare, γδ T-ALL accounts for up to 10% of T-ALL cases, and this is significantly higher than the proportion (1%) of γδ thymocytes in the healthy thymus. This raises the possibility that γδ thymocytes have increased potential for malignant transformation.[10,11,45] A possible contributor to this phenomenon could be CD27 costimulation, as it increases CK2 activity (synergistically with TCR stimulation) in γδ T-ALL cells expressing high levels of CD27. Of note, the importance of CK2 in T-cell biology is also underscored by its modulation by the inhibitory receptor PD-1 that decreases CK2 activity and AKT signaling in $CD4^+$ T cells.[35]

The ability of CK2 to affect AKT signaling was previously reported in T-ALL.[14,18,19] Here we showed for the first time that a CK2-AKT link exists in γδ thymocytes and γδ T-ALL cells. Most important, we considerably extended previous knowledge by demonstrating that AKT is essential for CK2-mediated effects: (1) chemical AKT inhibition (with MK-2206) mimicked CK2 inhibition (with CX-4945); (2) the latter extinguished AKT signaling (AKT phosphorylation and downstream effects); and (3) ectopic expression of a constitutively active form of AKT partially rescued the apoptosis because of CK2 inhibition. These results suggest that, although AKT activation is not sufficient to fully mimic CK2 activity, it is absolutely required for CK2-mediated effects in γδ T cells.

Maximal AKT activation requires phosphorylation at Ser129 (as reported in our western blot analyses) by CK2 both *in vitro* and *in vivo*.[46,47] Activated AKT promotes cell survival through direct phosphorylation of anti-apoptotic molecules, or indirectly through the transcriptional activation of anti-apoptotic genes and increased metabolic capacity.[48,49] AKT inhibits GSK3β activity[50] by direct phosphorylation of an N-terminal regulatory serine residue, allowing glycogen and protein synthesis.[51] Inhibition of CK2 activity in γδ thymocytes or γδ T-ALL cells abrogated the AKT/GSK3β signaling pathway and had a major impact on cell survival and proliferation. Interestingly, the strong dependence on AKT may be specific for human γδ T cells, as AKT-deficient mice were reported to have a normal γδ T-cell pool in the periphery.[52]

Overall, our demonstration of the high sensitivity γδ T-ALL cells to CK2 inhibition with CX-4945 *in vitro* and *in vivo* clearly supports its use for γδ T-ALL treatment. Importantly, CX-4945 is currently in phase II clinical trials in patients with multiple myeloma or advanced solid cancers.[27]

Finally, our study has implications, not only for hematology, but also for cancer immunotherapy, as γδ T cells are known to play important roles in protective (antitumor) responses.[53] In particular, we have recently documented the potent antileukemia properties of $V\delta1^+$ γδ T cells expressing natural cytotoxicity receptors.[9] The success of their clinical application, particularly in adoptive cell therapy, will strongly depend on the capacity to survive *ex vivo*

TCR-mediated activation and *in vivo* establishment and expansion. We thus believe an increased knowledge of the molecular determinants of γδ T-cell survival, as disclosed here for CK2, will be key to optimize their performance in cancer immunotherapy.

CONFLICT OF INTEREST

BSS is co-founder and share holder of Lymphact SA. The other authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

STR planned and performed the experiments and wrote the paper; MT planned and performed some experiments; JCR and EM helped to plan and/or perform the experiments; BS-S and JTB supervised the project, planned experiments and wrote the paper.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)