

## ORIGINAL ARTICLE

Casein kinase 2 controls the survival of normal thymic and leukemic  $\gamma\delta$  T cells via promotion of AKT signalingST Ribeiro<sup>1</sup>, M Tesio<sup>2,3</sup>, JC Ribot<sup>1,3</sup>, E Macintyre<sup>2</sup>, JT Barata<sup>1</sup> and B Silva-Santos<sup>1</sup>

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  $\gamma\delta$  T-cell lineage.  $\gamma\delta$  thymocytes display higher (and T-cell receptor inducible) CK2 activity than their  $\alpha\beta$  counterparts, and are strikingly sensitive to death upon CK2 inhibition. Mechanistically, we show that CK2 regulates the pro-survival AKT signaling pathway in  $\gamma\delta$  thymocytes and, importantly, also in  $\gamma\delta$  T-cell acute lymphoblastic leukemia (T-ALL) cells. When compared with healthy thymocytes or leukemic  $\alpha\beta$  T cells,  $\gamma\delta$  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  $\gamma\delta$  T-ALL. These data identify CK2 as a novel survival determinant of both healthy and leukemic  $\gamma\delta$  T cells, and may thus greatly impact their therapeutic manipulation.

Leukemia (2017) 31, 1603–1610; doi:10.1038/leu.2016.363

## 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  $\alpha\beta$  or a  $\gamma\delta$  TCR.<sup>1</sup> The expression of a pre-TCR composed of TCR $\beta$  and the invariant pT $\alpha$  chain in  $\alpha\beta$  thymocyte progenitors results in a massive proliferative burst (' $\beta$ -selection') that dictates that  $\alpha\beta$  T cells largely outnumber their  $\gamma\delta$  counterparts. Likely a consequence, although significant progress has been made in our understanding of human  $\alpha\beta$  T-cell development, the molecular determinants of  $\gamma\delta$  thymocytes remain poorly characterized.<sup>1</sup>

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

$\gamma\delta$  thymocytes can themselves transform into leukemic cells, causing a rare (< 10% of all cases) form of T-ALL with distinctive clinical features.<sup>10–12</sup> Given that malignant  $\gamma\delta$  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,<sup>13,14</sup> multiple myeloma,<sup>15</sup> B-ALL,<sup>16,17</sup> T-ALL<sup>18,19</sup> and other hematological disorders (reviewed in Piazza *et al.*<sup>20</sup>).

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- $\kappa$ B transcription and the DNA damage response.<sup>20</sup> CK2 displays pro-survival and anti-apoptotic functions that were described in several cancer cell types. CK2 is frequently over-expressed or hyperactivated in both solid tumors and hematological malignancies, thus making it a promising target for cancer treatment.<sup>20</sup> 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<sup>+</sup> T-cell activation and differentiation into T helper type 2 (Th2) or Th17 cells,<sup>21</sup> whereas the genetic deletion of CK2 in CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells abolished their suppressive activity against allergy-promoting Th2 cells.<sup>22</sup> 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  $\gamma\delta$  thymocytes and  $\gamma\delta$  T-ALL cells. We analyzed CK2 activity in  $\gamma\delta$  versus  $\alpha\beta$  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  $\gamma\delta$  T-ALL.

<sup>1</sup>Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal and <sup>2</sup>Institut Necker Enfants Malades, Hôpital Necker-Enfants Malades, APHP et Université Paris, Paris, France. Correspondence: Professor B Silva-Santos, Instituto de Medicina Molecular, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal. E-mail: bssantos@medicina.ulisboa.pt

<sup>3</sup>These two authors contributed equally to this work.

Received 17 September 2015; revised 12 October 2016; accepted 18 November 2016; accepted article preview online 30 November 2016; advance online publication, 16 December 2016

## 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 consent 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. TCR $\gamma\delta$ -positive T cells were isolated (to >97% purity) by magnetic positive selection; TCR $\alpha\beta$ -positive T cells were isolated (to >96% purity) by magnetic positive selection from the TCR $\gamma\delta$ -negative fraction (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were used as fresh or, when indicated, cells were cultured at 37 °C with 5% CO<sub>2</sub> in complete RPMI-1640 as previously described<sup>23</sup> 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.<sup>24</sup> To increase the percentage of transduced cells for the following experiments, GFP<sup>+</sup> cells were sorted ( $\approx$ 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 pan $\alpha\beta$ TCR (IP26) from eBioscience (San Diego, CA, USA); CD28 (CD28.2), CD8 (SK1), CD45 (HI30), V $\delta$ 2 (B6), CD3 (OKT3), CD45RA (HI100), Annexin-V and 7-aminoactinomycin D (7-AAD) from Biotegend (San Diego, CA, USA); pany $\delta$ TCR (5A6.E9) from ThermoFisher (Rockford, IL, USA); V $\delta$ 1 (REA173) from Miltenyi Biotec; p-S129-AKT, AKT, p-S9-GSK3 $\beta$ , GSK3 $\beta$ , 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.<sup>25</sup> For cell cycle analysis, cells were stained for 1 h at 37 °C with 30  $\mu$ l 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.<sup>4</sup> 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: CK2 $\alpha$ -specific peptide, [ $\gamma$ -<sup>32</sup>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.<sup>14</sup> CK2 activity in  $\gamma\delta$ <sup>+</sup> and  $\alpha\beta$ <sup>+</sup> 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 immunoblotting as previously described.<sup>14</sup> 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 were 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. TCR $\alpha\beta$ - or TCR $\gamma\delta$ -positive T-ALL cells obtained from patient diagnostic were injected into 8–12-week-old NSG mice (1  $\times$  10<sup>6</sup> cells/mouse, tail vein injections). Mice were monitored weekly by flow cytometry for human leukemic load (hCD7<sup>+</sup>, hCD45<sup>+</sup> cells) in peripheral blood. Mice were killed when terminally ill and blast cells from bone marrow were collected. For the *in vivo* experiment of  $\gamma\delta$  T-ALL treatment, 10–12-week-old NRG5 mice were injected subcutaneously in the right flank with 2  $\times$  10<sup>6</sup> PEER cells resuspended in 100  $\mu$ l of phosphate-buffered saline. At day 20, all mice presented palpable tumors (100–150 mm<sup>3</sup>) and were randomly distributed into two groups ( $n \geq 3$ ). 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.<sup>26</sup> Mice were monitored daily and weighed frequently. Tumors were measured every 2 days with a caliper and tumor volume was calculated (volume = (length  $\times$  width)<sup>2</sup>/2). At day 13, after starting the treatment, all mice were killed (an ethical requirement for the control group, when tumor reached 2000 mm<sup>3</sup>).

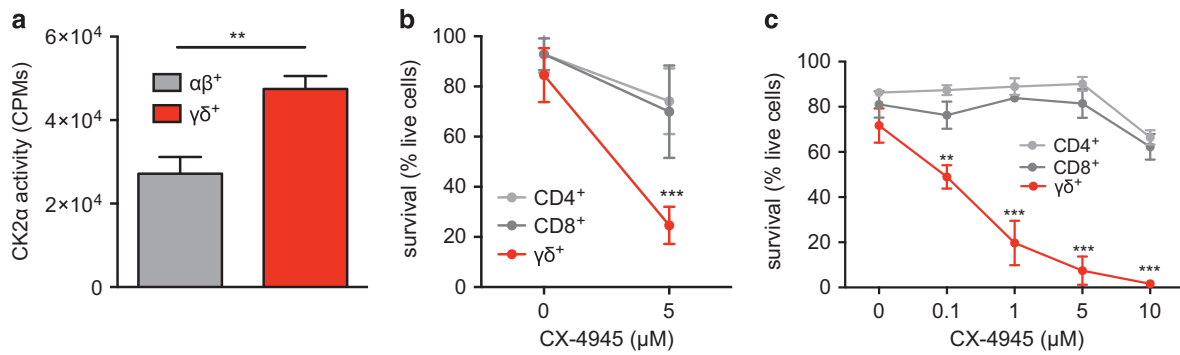
### Statistical analysis

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  $\gamma\delta$  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 TCR $\gamma\delta$ <sup>+</sup> or TCR $\alpha\beta$ <sup>+</sup> cells, and unexpectedly found twofold higher activity in  $\gamma\delta$  thymocytes relative to their  $\alpha\beta$  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.<sup>27</sup> Flow cytometry analysis of Annexin-V/7-AAD-stained cells revealed increased apoptosis of  $\gamma\delta$  compared with CD4<sup>+</sup> and CD8<sup>+</sup>  $\alpha\beta$  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  $\gamma\delta$  thymocytes were highly susceptible to apoptosis upon CK2 inhibition in a dose dependent-manner (Figure 1c). In contrast, as previously reported,<sup>18</sup> CD4<sup>+</sup> or CD8<sup>+</sup>  $\alpha\beta$  thymocyte survival was not significantly affected following CK2 inhibition (Figure 1c).



**Figure 1.** Human  $\gamma\delta$  thymocytes have enhanced CK2 activity and are highly sensitive to CX-4945. (a) *In vitro* CK2 $\alpha$  activity (kinase assay) in freshly isolated human thymic  $\gamma\delta$  and  $\alpha\beta$  T-cells ( $2 \times 10^6$  cells per assay). CPM, counts per min. (b) Survival (% of live cells) of human thymic  $\gamma\delta$ ,  $CD4^+$  and  $CD8^+$  T cells following 24 h of incubation with 5  $\mu$ M of the CK2 inhibitor, CX-4945, analyzed by flow cytometry using Annexin-V/7-AAD staining. (c) Survival (% of live cells) of human thymic  $\gamma\delta$ ,  $CD4^+$  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  $\gamma\delta$  or  $\alpha\beta$  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  $\gamma\delta$  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,<sup>28,29</sup> we also tested another CK2 inhibitor, 4,5,6,7-tetrabromobenzotriazole (TBB); TG-003 that specifically targets CLK2,<sup>28,30</sup> and Harmine that selectively inhibits DYRK1A.<sup>31</sup> Importantly, only the other CK2 inhibitor, TBB, reproduced the effects of CX-4945, whereas TG-003 and Harmine failed to affect  $\gamma\delta$  thymocyte survival (Supplementary Figure S1). These results strongly suggest that the physiological target of CX-4945 in  $\gamma\delta$  thymocytes is CK2.

#### CK2 activity in $\gamma\delta$ thymocytes is modulated by TCR stimulation and promotes AKT signaling

We next asked which signals regulated CK2 activity in  $\gamma\delta$  thymocytes. Very few studies have documented CK2 modulation by physiological stimuli in T cells.<sup>21,32</sup> When we stimulated (for 6 h) isolated thymocyte subsets via the TCR complex using agonist anti-CD3 $\epsilon$  antibodies, we observed an approximately threefold enhancement of CK2 activity selectively in  $\gamma\delta$  thymocytes (Figure 2a). We also tested the impact of costimulation, particularly through CD27 that we have shown to play a major role in  $\gamma\delta$  T-cell development and expansion.<sup>4,33,34</sup> 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  $\gamma\delta$  thymocytes is modulated primarily by TCR signals.

To gain insight into the downstream effects of CK2 signaling and its inhibition in  $\gamma\delta$  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  $\alpha\beta$  T lymphocytes.<sup>18,35</sup> 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  $\gamma\delta$  but not  $\alpha\beta$  thymocytes, as shown by the phosphorylation of AKT and its downstream targets glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) 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  $\gamma\delta$  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  $\gamma\delta$

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 $\gamma\delta$ T-ALL

We next asked how CK2 activity would affect  $\gamma\delta$  T-ALL. First, we compared CK2 activity in normal  $\gamma\delta$  and  $\alpha\beta$  thymocytes versus  $\gamma\delta$  and  $\alpha\beta$  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,  $\alpha\beta$  T-ALL cells displayed higher levels of CK2 activity than  $\alpha\beta$  thymocytes (Figure 3). Notably, we detected markedly higher CK2 activity in  $\gamma\delta$  T-ALL cells as compared with healthy thymocytes and  $\alpha\beta$  T-ALL blasts (Figure 3). Moreover, the  $\gamma\delta$  T-ALL cell line PEER reproduced the very high CK2 activity observed in  $\gamma\delta$  T-ALL blasts (Figure 3), making it a good model for further biochemical and functional CK2 tests in  $\gamma\delta$  T-ALL.

The CK2 inhibitor, CX-4945, suppressed CK2 activity in  $\gamma\delta$  T-ALL cells in a dose-dependent manner (Figure 4a). As with healthy  $\gamma\delta$  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  $\gamma\delta$  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  $\gamma\delta$  T-ALL cells (Figure 4b), in contrast with  $\gamma\delta$  thymocytes (Figure 2a). Of note, PEER cells are  $CD27^+ V\delta 1^+$   $\gamma\delta$  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  $\gamma\delta$  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.<sup>37</sup> Cells expressing myristoylated AKT displayed high levels of AKT phosphorylation that were insensitive to CK2 inhibition (Figure 4e). Importantly, AKT hyperactivation partially rescued  $\gamma\delta$  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.

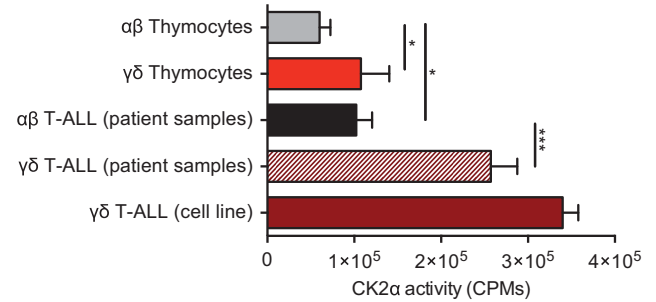
$\gamma\delta$  T-ALL cells are highly sensitive to CK2 inhibition *in vitro* and *in vivo*

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

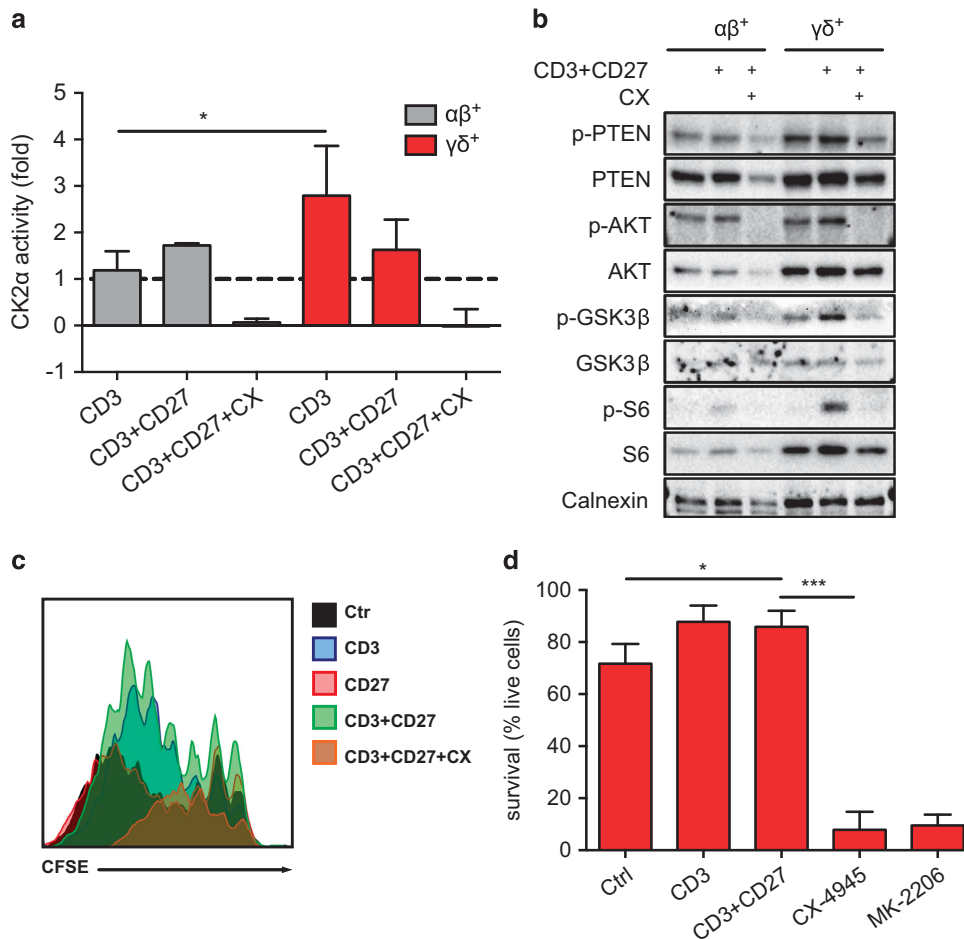
Upon 48 h of *in vitro* treatment with CX-4945, we observed increased cell apoptosis in primary  $\gamma\delta$  T-ALL samples compared with  $\alpha\beta$  T-ALL cells (Figure 5a). We performed a more detailed test with the T-ALL cell lines, at various time points of incubation with CX-4945, and found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

Finally, the increased sensitivity of  $\gamma\delta$  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

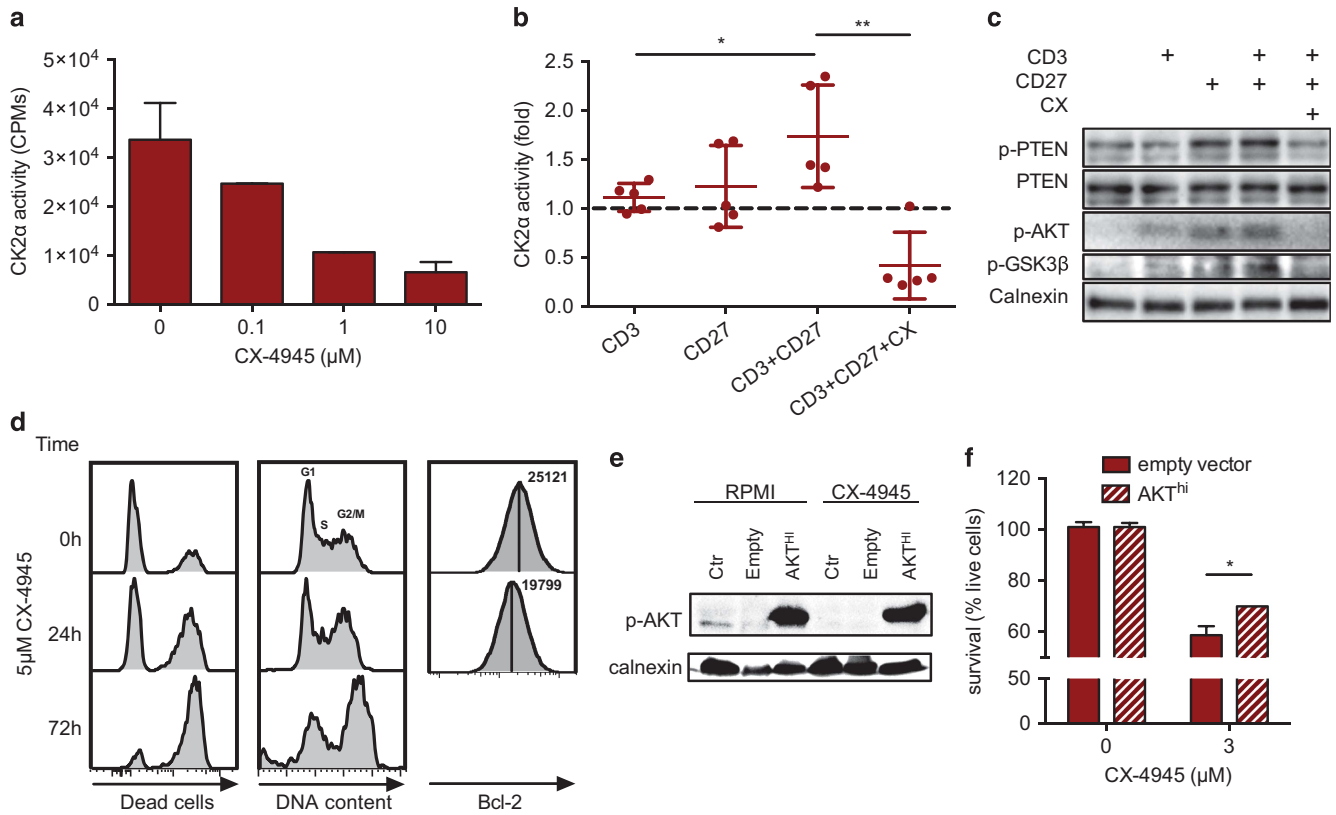
$\gamma\delta$  T-ALL by injecting  $2 \times 10^6$  PEER cells subcutaneously in immune-deficient NRG5 (NOD-Rag1<sup>null</sup> IL2Rgamma<sup>null</sup>) mice. After the detection of palpable tumor, mice were equally distributed



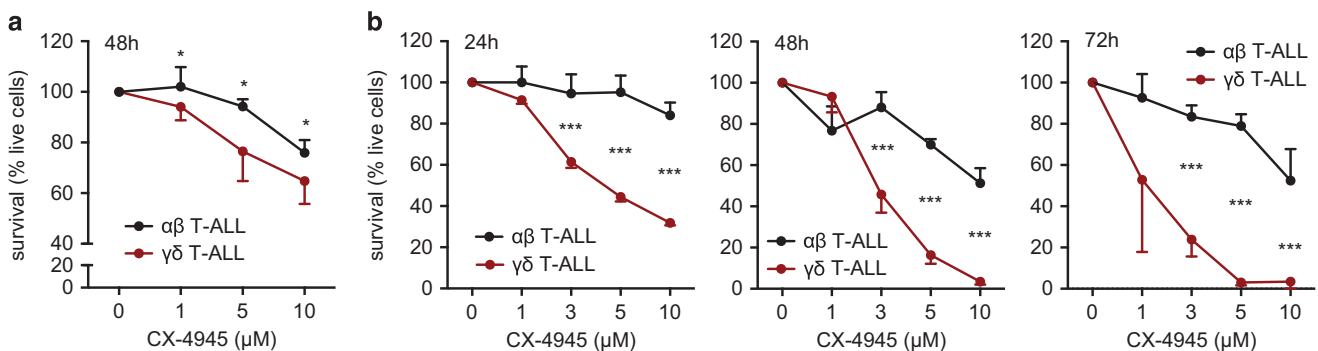
**Figure 3.**  $\gamma\delta$  T-ALL cells display higher CK2 activity than  $\alpha\beta$  counterparts. *In vitro* CK2 $\alpha$  activity (kinase assay;  $6.6 \times 10^6$  cells per assay) in freshly isolated  $\gamma\delta$  ( $n=4$ ) and  $\alpha\beta$  ( $n=4$ ) thymocyte samples;  $\gamma\delta$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



**Figure 2.** CK2 activity in  $\gamma\delta$  thymocytes is modulated by TCR stimulation and activates AKT signaling. **(a)** *In vitro* CK2 $\alpha$  activity in sorted  $\gamma\delta$  and  $\alpha\beta$  thymocytes ( $2 \times 10^6$  cells per sample) after 6 h of stimulation with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27) or plus  $5 \mu\text{M}$  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  $\gamma\delta$  and  $\alpha\beta$  thymocytes ( $1 \times 10^6$  cells per sample) stimulated as in **(a)**. **(c)** Proliferation (CFSE dilution assay) of  $\gamma\delta$  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  $5 \mu\text{M}$  CX-4945 (CD3+CD27+CX). **(d)** Survival (% of live cells) of  $\gamma\delta$  thymocytes after 7 days of stimulation (or not, Ctrl for control) with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27), plus  $5 \mu\text{M}$  of CX-4945 or  $10 \mu\text{M}$  of MK-2206. Data in this figure are representative of at least three independent experiments; \* $P < 0.05$ , \*\*\* $P < 0.001$  (*T*-test).



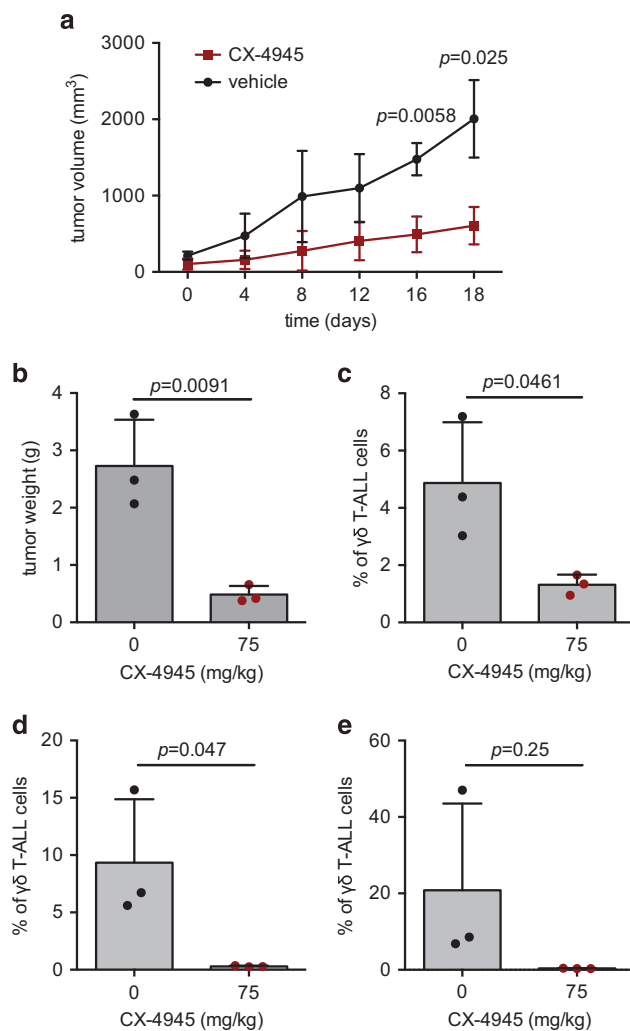
**Figure 4.** CK2 activity in  $\gamma\delta$  T-ALL cells is potentiated by CD27 costimulation and promotes AKT signaling. **(a)** CK2 $\alpha$  activity in the  $\gamma\delta$  T-ALL cell line, PEER ( $2 \times 10^6$  cells per condition), after 6 h of treatment with indicated concentrations of CX-4945. **(b)** CK2 $\alpha$  activity in lysates from  $\gamma\delta$  T-ALL (PEER) cells ( $2 \times 10^6$  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  $\gamma\delta$  T-ALL (PEER) cells treated for 6 h with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27) or plus 5  $\mu\text{M}$  CX-4945 (CD3+CD27+CX). Data are representative of five independent experiments. **(d)** Flow cytometry analysis of apoptosis (Annexin-V<sup>+</sup>; left panel), cell cycle/DNA staining (middle panel) and intracellular Bcl-2 protein staining (right panel; values indicate mean fluorescence intensity (MFI)) of  $\gamma\delta$  T-ALL (PEER) cells treated with CX-4945 (5  $\mu\text{M}$ ) 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<sup>hi</sup>) and treated with 3  $\mu\text{M}$  CX-4945 or vehicle (*T*-test,  $*P < 0.05$ ).



**Figure 5.**  $\gamma\delta$  T-ALL cells are more susceptible than  $\alpha\beta$  T-ALL to apoptosis induced by CX-4945. Flow cytometry analysis of the survival (Annexin-V/7-AAD staining) of **(a)**  $\gamma\delta$  ( $n = 5$ ) and  $\alpha\beta$  ( $n = 5$ ) T-cell blast samples (obtained from T-ALL patients and expanded in NSG mice) or **(b)**  $\gamma\delta$  (PEER) or  $\alpha\beta$  (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  $\gamma\delta$  T-ALL.



**Figure 6.** CX-4945 treatment inhibits  $\gamma\delta$  T-ALL growth *in vivo*. (a) Tumor volume following injection of  $2 \times 10^6$  PEER  $\gamma\delta$  T-ALL cells subcutaneously into NRG5 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<sup>+</sup> CD7<sup>+</sup>  $\gamma\delta$  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,<sup>38</sup> but few reports have demonstrated its relevance in physiological conditions.<sup>32,39</sup> 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,<sup>40</sup> for CD4<sup>+</sup> T-cell activation and differentiation into Th2 or Th17 cells<sup>21</sup> and for the suppressive function of CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells against allergy-promoting Th2 cells.<sup>22</sup> 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  $\gamma\delta$  lineage of human thymocytes. These display approximately twofold higher CK2 activity and are strikingly more sensitive to CK2 inhibition than their  $\alpha\beta$  counterparts. As we show that TCR stimulation increases (~2.5-fold) CK2 activity in  $\gamma\delta$  thymocytes, we may speculate that the different basal levels of CK2 activity in

$\gamma\delta$  versus  $\alpha\beta$  thymocytes are because of stronger TCR signals received during their development. It is well established that strong TCR signaling favors  $\gamma\delta$  over  $\alpha\beta$  T-cell lineage commitment<sup>1,41–43</sup> and further affects subsequent  $\gamma\delta$  thymocyte development.<sup>44</sup> In this line of reasoning, the high CK2 activity in agonist-selected  $\gamma\delta$  thymocytes could be an important pro-survival mechanism to counteract the activation-induced cell death underlying thymocyte negative selection.

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

Although rare,  $\gamma\delta$  T-ALL accounts for up to 10% of T-ALL cases, and this is significantly higher than the proportion (1%) of  $\gamma\delta$  thymocytes in the healthy thymus. This raises the possibility that  $\gamma\delta$  thymocytes have increased potential for malignant transformation.<sup>10,11,45</sup> A possible contributor to this phenomenon could be CD27 costimulation, as it increases CK2 activity (synergistically with TCR stimulation) in  $\gamma\delta$  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<sup>+</sup> T cells.<sup>35</sup>

The ability of CK2 to affect AKT signaling was previously reported in T-ALL.<sup>14,18,19</sup> Here we showed for the first time that a CK2-AKT link exists in  $\gamma\delta$  thymocytes and  $\gamma\delta$  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  $\gamma\delta$  T cells.

Maximal AKT activation requires phosphorylation at Ser129 (as reported in our western blot analyses) by CK2 both *in vitro* and *in vivo*.<sup>46,47</sup> 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.<sup>48,49</sup> AKT inhibits GSK3 $\beta$  activity<sup>50</sup> by direct phosphorylation of an N-terminal regulatory serine residue, allowing glycogen and protein synthesis.<sup>51</sup> Inhibition of CK2 activity in  $\gamma\delta$  thymocytes or  $\gamma\delta$  T-ALL cells abrogated the AKT/GSK3 $\beta$  signaling pathway and had a major impact on cell survival and proliferation. Interestingly, the strong dependence on AKT may be specific for human  $\gamma\delta$  T cells, as AKT-deficient mice were reported to have a normal  $\gamma\delta$  T-cell pool in the periphery.<sup>52</sup>

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

Finally, our study has implications, not only for hematology, but also for cancer immunotherapy, as  $\gamma\delta$  T cells are known to play important roles in protective (antitumor) responses.<sup>53</sup> In particular, we have recently documented the potent antileukemia properties of V $\delta$ 1<sup>+</sup>  $\gamma\delta$  T cells expressing natural cytotoxicity receptors.<sup>9</sup> 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  $\gamma\delta$  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.

### ACKNOWLEDGEMENTS

We thank Miguel Abecasis and Rui Anjos (Hospital de Santa Cruz) and Ana E Sousa (IMM) for provision of the pediatric thymic samples; Paul Coffey for the myrPKB/AKT construct; and Francisco Caiado, Daniel Correia, Alice Melão, Daniel Ribeiro, Natacha Sousa, Hiroshi Kubo, Helena Nunes Cabaço, Pedro Oliveira and Tiago Amado (all IMM) for technical assistance. This work was supported by project grants from the European Research Council, CoG\_646701 (to BSS), Fundação para a Ciência e Tecnologia (FCT), PTDC/DTP-PIC/4931/2014 (to BSS), EXPL/IMI-IMU/0170/2013 (to JCR), PTDC/SAU-ONC/113202/2009 and PTDC/SAU-ONC/122428/2010 (to JTB); and ARC-Association pour la Recherche sur le Cancer (to MT); and by individual fellowships from FCT, SFRH/BD/84123/2012 (to STR) and IF/00013/2014 (to JCR).

### 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.

### REFERENCES

- Ciofani M, Zúñiga-Pflücker JC. Determining  $\gamma\delta$  versus  $\alpha\beta$  T cell development. *Nat Rev Immunol* 2010; **10**: 657–663.
- Silva-Santos B, Pennington DJ, Hayday AC. Lymphotoxin-mediated regulation of gammadelta cell differentiation by alphabeta T cell progenitors. *Science* 2005; **307**: 925–928.
- Melichar HJ, Narayan K, Der SD, Hiraoka Y, Gardiol N, Jeannot G *et al*. Regulation of gammadelta versus alphabeta T lymphocyte differentiation by the transcription factor SOX13. *Science* 2007; **315**: 230–233.
- Ribot JC, DeBarros A, Pang DJ, Neves JF, Peperzak V, Roberts SJ *et al*. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat Immunol* 2009; **10**: 427–436.
- Lauritsen JPH, Wong GW, Lee S-Y, Lefebvre JM, Ciofani M, Rhodes M *et al*. Marked induction of the helix-loop-helix protein Id3 promotes the  $\gamma\delta$  T cell fate and renders their functional maturation Notch independent. *Immunity* 2009; **31**: 565–575.
- Malhotra N, Narayan K, Cho OH, Sylvia KE, Yin C, Melichar H *et al*. A network of high-mobility group box transcription factors programs innate interleukin-17 production. *Immunity* 2013; **38**: 681–693.
- Schmolka N, Wencker M, Hayday AC, Silva-Santos B. Epigenetic and transcriptional regulation of  $\gamma\delta$  T cell differentiation: programming cells for responses in time and space. *Semin Immunol* 2015; **27**: 19–25.
- Ribot JC, Ribeiro ST, Correia D V, Sousa AE, Silva-Santos B. Human  $\gamma\delta$  thymocytes are functionally immature and differentiate into cytotoxic type 1 effector T cells upon IL-2/IL-15 signaling. *J Immunol* 2014; **192**: 2237–2243.
- Almeida AR, Correia DV, Fernandes-Platzgummer A, da Silva CL, Gomes da Silva M, Anjos DR *et al*. Delta One T cells for immunotherapy of chronic lymphocytic leukemia: clinical-grade expansion/ differentiation and preclinical proof-of-concept. *Clin Cancer Res* 2016; **22**: 5795–5804.
- Matos DM, Rizzatti EG, Fernandes M, Buccheri V, Falcão RP. Gammadelta and alphabeta T-cell acute lymphoblastic leukemia: comparison of their clinical and immunophenotypic features. *Haematologica* 2005; **90**: 264–266.
- Schott G, Sperling C, Schrappe M, Räte R, Martin M, Meyer U *et al*. Immunophenotypic and clinical features of T-cell receptor gammadelta+ T-lineage acute lymphoblastic leukaemia. *Br J Haematol* 1998; **101**: 753–755.
- van Grotel M, Meijerink JPP, van Wering ER, Langerak AW, Beverloo HB, Buijs-Gladdines JGC A M *et al*. Prognostic significance of molecular-cytogenetic abnormalities in pediatric T-ALL is not explained by immunophenotypic differences. *Leukemia* 2008; **22**: 124–131.
- Shehata M, Schnabl S, Demirtas D, Hilgarth M, Hubmann R, Ponath E *et al*. Reconstitution of PTEN activity by CK2 inhibitors and interference with the PI3-K/Akt cascade counteract the antiapoptotic effect of human stromal cells in chronic lymphocytic leukemia. *Blood* 2010; **116**: 2513–2521.

- Martins LR, Lúcio P, Silva MC, Anderes KL, Gameiro P, Silva MG *et al*. Targeting CK2 overexpression and hyperactivation as a novel therapeutic tool in chronic lymphocytic leukemia. *Blood* 2010; **116**: 2724–2731.
- Piazza FA, Ruzzene M, Gurrieri C, Montini B, Bonanni L, Chioetto G *et al*. Multiple myeloma cell survival relies on high activity of protein kinase CK2. *Blood* 2006; **108**: 1698–1707.
- Margarida Gomes A, Soares MVD, Ribeiro P, Caldas J, Póvoa V, Martins LR *et al*. Adult B-cell acute lymphoblastic leukemia cells display decreased PTEN activity and constitutive hyperactivation of PI3K/Akt pathway despite high PTEN protein levels. *Haematologica* 2014; **99**: 1062–1068.
- Song C, Gowda C, Pan X, Ding Y, Tong Y, Tan B *et al*. Targeting casein kinase II restores Ikaros tumor suppressor activity and demonstrates therapeutic efficacy in high-risk leukemia. *Blood* 2015; **126**: 1813–1823.
- Silva A, Yunes JA, Cardoso BA, Martins LR, Jotta PY, Abecasis M *et al*. PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest* 2008; **118**: 3762–3774.
- Buontempo F, Orsini E, Martins LR, Antunes I, Lonetti A, Chiarini F *et al*. Cytotoxic activity of the casein kinase 2 inhibitor CX-4945 against T-cell acute lymphoblastic leukemia: targeting the unfolded protein response signaling. *Leukemia* 2014; **28**: 543–553.
- Piazza F, Manni S, Ruzzene M, Pinna LA, Gurrieri C, Semenzato G. Protein kinase CK2 in hematologic malignancies: reliance on a pivotal cell survival regulator by oncogenic signaling pathways. *Leukemia* 2012; **26**: 1174–1179.
- Sestero CM, McGuire DJ, De Sarno P, Brantley EC, Soldevila G, Axtell RC *et al*. CD5-dependent CK2 activation pathway regulates threshold for T cell anergy. *J Immunol* 2012; **189**: 2918–2930.
- Ulges A, Klein M, Reuter S, Gerlitzki B, Hoffmann M, Grebe N *et al*. Protein kinase CK2 enables regulatory T cells to suppress excessive TH2 responses in vivo. *Nat Immunol* 2015; **16**: 267–275.
- Ribot JC, DeBarros A, Mancio-Silva L, Pamplona A, Silva-Santos B. B7-CD28 costimulatory signals control the survival and proliferation of murine and human  $\gamma\delta$  T cells via IL-2 production. *J Immunol* 2012; **189**: 1202–1208.
- Buitenhuis M, Van Der Linden E, Ulfman LH, Hofhuis FM, Bierings MB, Coffey PJ. Protein kinase B (PKB/c-akt) regulates homing of hematopoietic progenitors through modulation of their adhesive and migratory properties. *Blood* 2010; **116**: 2373–2384.
- Martins LR, Perera Y, Lúcio P, Silva MG, Perea SE, Barata JT. Targeting chronic lymphocytic leukemia using CIGB-300, a clinical-stage CK2-specific cell-permeable peptide inhibitor. *Oncotarget* 2014; **5**: 258–263.
- Siddiqui-Jain A, Drygin D, Streiner N, Chua P, Pierre F, O'Brien SE *et al*. CX-4945, an orally bioavailable selective inhibitor of protein kinase CK2, inhibits pro-survival and angiogenic signaling and exhibits antitumor efficacy. *Cancer Res* 2010; **70**: 10288–10298.
- Cozza G, Pinna LA, Moro S. Protein kinase CK2 inhibitors: a patent review. *Expert Opin Ther Pat* 2012; **22**: 1081–1097.
- Kim H, Choi K, Kang H, Lee SY, Chi SW, Lee MS *et al*. Identification of a novel function of CX-4945 as a splicing regulator. *PLoS One* 2014; **9**: 1–8.
- Kim H, Lee K-S, Kim A-K, Choi M, Choi K, Kang M *et al*. A chemical with proven clinical safety rescues Down-syndrome-related phenotypes in through DYRK1A inhibition. *Dis Model Mech* 2016; **9**: 839–848.
- Muraki M, Ohkawara B, Hosoya T, Onogi H, Koizumi J, Koizumi T *et al*. Manipulation of Alternative Splicing by a Newly Developed Inhibitor of Clks. *J Biol Chem* 2004; **279**: 24246–24254.
- Adayev T, Wegiel J, Hwang YW. Harmine is an ATP-competitive inhibitor for dual-specificity tyrosine phosphorylation-regulated kinase 1A (Dyrk1A). *Arch Biochem Biophys* 2011; **507**: 212–218.
- Barata JT. The impact of PTEN regulation by CK2 on PI3K-dependent signaling and leukemia cell survival. *Adv Enzyme Regul* 2011; **51**: 37–49.
- Ribeiro ST, Ribot JC, Silva-Santos B. Five layers of receptor signaling in  $\gamma\delta$  T-cell differentiation and activation. *Front Immunol* 2015; **6**: 1–9.
- DeBarros A, Chaves-Ferreira M, D'Orey F, Ribot JC, Silva-Santos B. CD70–CD27 interactions provide survival and proliferative signals that regulate T cell receptor-driven activation of human  $\gamma\delta$  peripheral blood lymphocytes. *Eur J Immunol* 2011; **41**: 195–201.
- Patsoukis N, Li L, Sari D, Petkova V, Boussiotis VA. PD-1 increases PTEN phosphatase activity while decreasing PTEN protein stability by inhibiting CK2. *Mol Cell Biol* 2013; **33**: 3091–3098.
- Pal SK, Reckamp K, Yu H, Figlin RA. Akt inhibitors in clinical development for the treatment of cancer. *Expert Opin Investig Drugs* 2010; **19**: 1355–1366.
- Buitenhuis M, Verhagen LP, van Deutekom HWM, Castor A, Verploegen S, Koenderman L *et al*. Protein kinase B (c-akt) regulates hematopoietic lineage choice decisions during myelopoiesis. *Blood* 2008; **111**: 112–121.



- 38 Trembley JH, Wang G, Unger G, Slaton J, Ahmed K. CK2: A key player in cancer biology. *Cell Mol Life Sci* 2009; **66**: 1858–1867.
- 39 Litchfield DW. Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J* 2003; **369**: 1–15.
- 40 Koch S, Capaldo CT, Hilgarth RS, Fournier B, Parkos CA, Nusrat A. Protein kinase CK2 is a critical regulator of epithelial homeostasis in chronic intestinal inflammation. *Mucosal Immunol* 2012; **6**: 136–145.
- 41 Haks MC, Lefebvre JM, Lauritsen JPH, Carleton M, Rhodes M, Miyazaki T *et al*. Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity* 2005; **22**: 595–606.
- 42 Hayes SM, Li L, Love PE. TCR signal strength influences alphabeta/gammadelta lineage fate. *Immunity* 2005; **22**: 583–593.
- 43 Pennington DJ, Silva-Santos B, Hayday AC.  $\gamma\delta$  T cell development—having the strength to get there. *Curr Opin Immunol* 2005; **17**: 108–115.
- 44 Muñoz-Ruiz M, Ribot JC, Grosso AR, Gonçalves-Sousa N, Pamplona A, Pennington DJ *et al*. TCR signal strength controls thymic differentiation of discrete proinflammatory  $\gamma\delta$  T cell subsets. *Nat Immunol* 2016; **17**: 721–728.
- 45 Macintyre EA, Salloum E, Sigaux F. Comparison of alpha beta and gamma delta expressing CD3+ acute lymphoblastic leukemias. *Nouv Rev Fr Hématol* 1990; **32**: 95–99.
- 46 Di Maira G, Salvi M, Arrigoni G, Marin O, Sarno S, Brustolon F *et al*. Protein kinase CK2 phosphorylates and upregulates Akt/PKB. *Cell Death Differ* 2005; **12**: 668–677.
- 47 Ponce DP, Maturana JL, Cabello P, Yefi R, Niechi I, Silva E *et al*. Phosphorylation of AKT/PKB by CK2 is necessary for the AKT-dependent up-regulation of  $\beta$ -catenin transcriptional activity. *J Cell Physiol* 2011; **226**: 1953–1959.
- 48 Plas DR, Thompson CB. Akt-dependent transformation: there is more to growth than just surviving. *Oncogene* 2005; **24**: 7435–7442.
- 49 Risso G, Blaustein M, Pozzi B, Mammi P, Srebrow A. Akt/PKB: one kinase, many modifications. *Biochem J* 2015; **468**: 203–214.
- 50 Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995; **378**: 785–789.
- 51 Piazza F, Manni S, Semenzato G. Novel players in multiple myeloma pathogenesis: role of protein kinases CK2 and GSK3. *Leuk Res* 2013; **37**: 221–227.
- 52 Juntilla MM, Wofford JA, Birnbaum MJ, Rathmell JC, Koretzky GA. Akt1 and Akt2 are required for alphabeta thymocyte survival and differentiation. *Proc Natl Acad Sci USA* 2007; **104**: 12105–12110.
- 53 Silva-Santos B, Serre K, Norell H.  $\gamma\delta$  T cells in cancer. *Nat Rev Immunol* 2015; **15**: 683–691.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>

© The Author(s) 2017

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