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Optimal interleukin-7 receptor-mediated signaling, cell cycle progression and viability of T-cell acute lymphoblastic leukemia cells rely on casein kinase 2 activity

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ABSTRACT

Interleukin-7 and interleukin-7 receptor are essential for normal T-cell development and homeostasis, whereas excessive interleukin-7/interleukin-7 receptor-mediated signaling promotes leukemogenesis. The protein kinase, casein kinase 2, is overexpressed and hyperactivated in cancer, including T-cell acute lymphoblastic leukemia. Herein, we show that while interleukin-7 had a minor but significant positive effect on casein kinase 2 activity in leukemia T-cells, casein kinase 2 activity was mandatory for optimal interleukin-7/ interleukin-7 receptor-mediated signaling. Casein kinase 2 pharmacological inhibition impaired signal transducer and activator of transcription 5 and phosphoinositide 3-kinase/v-Akt murine thymoma viral oncogene homolog 1 pathway activation triggered by interleukin-7 or by mutational activation of interleukin-7 receptor. By contrast, forced expression of casein kinase 2 augmented interleukin-7 signaling in human embryonic kidney 293T cells reconstituted with the interleukin-7 receptor machinery. Casein kinase 2 inactivation prevented interleukin-7-induced B-cell lymphoma 2 upregulation, maintenance of mitochondrial homeostasis and viability of T-cell acute lymphoblastic leukemia cell lines and primary leukemia cells collected from patients at diagnosis. Casein kinase 2 inhibition further abrogated interleukin-7-mediated cell growth and upregulation of the transferrin receptor, and blocked cyclin A and E upregulation and cell cycle progression. Notably, casein kinase 2 was also required for the viability of mutant interleukin-7 receptor expressing leukemia T-cells. Overall, our study identifies casein kinase 2 as a major player in the effects of interleukin-7 and interleukin-7 receptor in T-cell acute lymphoblastic leukemia. This further highlights the potential relevance of targeting casein kinase 2 in this malignancy.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematological cancer that results from the transformation of thymic T-cell precursors and accounts for 10–15% of pediatric ALL cases. Although the 5-year event-free survival rate has significantly improved for these patients, reaching up to 80%, they still present an increased risk for early relapse with very poor prognosis.[1] Moreover, current intensive therapies have considerable long-term side effects. Thus, it is critical to better define the underlying mechanisms involved in leukemogenesis and resistance to treatment, in order to develop improved therapeutic strategies that minimize toxicities and the probability of relapse.

Interleukin-7 (IL-7) is a cytokine essential for normal T-cell development and homeostasis in humans and mice.[2,3] IL-7 is present in the microenvironments where T-cell precursors reside, and are secreted by a variety of cells, amongst which stro-

mal cells are involved, in the thymus and bone marrow. In the last few years several studies have provided new insights into the relevance of this cytokine and its receptor (IL-7R) for the development of autoimmune and chronic inflammatory diseases.[4] Moreover, activation of the IL-7/IL-7R signaling axis has been shown to contribute to T-cell leukemogenesis,[5-10] whereas IL-7 deficiency leads to decreased *in vivo* expansion of leukemia T-cells and delayed leukemia-associated death in mice transplanted with human T-ALL cells.[11] Notably, we and others revealed that *IL7R* (encoding the IL-7Rα subunit, also known as CD127) is a *bona fide* oncogene. Around 10% of pediatric T-ALL patients display *IL7R* gain-of-function mutations, which lead to constitutive activation of downstream signaling and subsequent promotion of cell transformation and tumorigenesis.[12-16]

Casein kinase 2 (CK2) is a ubiquitously expressed serine/threonine kinase, that is involved in the regulation of numerous cellular processes (e.g. cell cycle, gene expression and proliferation), through the modulation of the crosstalk between multiple signaling pathways.[17] Many of the CK2 described substrates are proteins involved in the regulation of cell survival, with compiled evidence that the reduction of CK2 activity or expression leads to cell death, in such a way that CK2 is considered to have mainly a pro-survival and proliferative function. In agreement with these features, CK2 is significantly and consistently overexpressed in solid[18] and hematological[19-22] tumor cells, including T-ALL.[23]

Primary T-ALL cells collected from diagnostic patients display basal hyperactivation of the PI3K/Akt signaling pathway.[23] Although gene inactivation of *PTEN*, the main negative regulator of the pathway, can occur in up to 25% of T-ALL cases,[23,24] PI3K/Akt signaling pathway activation results most frequently from PTEN post-translational inhibition mediated by oxidation *via* reactive oxygen species and by phosphorylation due to high CK2 activity in the leukemia cells.[23] More recently, it has been shown that CK2 also regulates the JAK/STAT pathway by interacting with JAKs, thereby facilitating the activation of STATs.[25] These observations highlight the ability of CK2 to positively modulate JAK/STAT and PI3K/Akt pathways in the context of cancer. Notably, PI3K/Akt/mTOR and JAK/STAT signaling pathways are also activated by IL-7, and have a pivotal role in leukemia development.[26] However, whether CK2 is involved in IL-7-mediated signaling, particularly in the context of T-cell leukemia, remains to be elucidated.

Although CK2 has constitutive kinase activity and is viewed as largely refractory to 'vertical' stimulation by growth factors, playing mostly a 'horizontal' role as a modulator of the activity of diverse signaling pathways,[27] there is evidence that CK2 can play an important function downstream from external stimuli.[28,29] In the study herein, we evaluated the possible involvement of CK2 in IL-7-mediated effects on T-ALL cells. Our results indicate that CK2 activity is essential for optimal IL-7/IL-7R-dependent signaling *via* PI3K/Akt and JAK/STAT pathways in leukemia T-cells. Moreover, inhibition of CK2 prevents IL-7/IL-7R-mediated viability and cell cycle progression of T-ALL cells. Our results indicate that CK2 partakes in T-cell leukemia development, not only *via* its basal impact on key oncogenic signaling pathways, but also by being a major regulator of IL-7/IL-7R-mediated signaling in T-ALL.

Methods

Cells

Primary leukemia cells were obtained from the bone marrow and/or peripheral blood of diagnostic pediatric T-ALL cases, and were classified according to the European Group for the Immunological Classification of Leukemias (EGIL) criteria[30] (Table 1). Informed consent was obtained in accordance with the Declaration of Helsinki and under the ethical review board approval of Instituto Português de Oncologia (Lisbon, Portugal). The IL-7–dependent cell line TAIL7, which shares significant similarities with primary leukemia samples,[31] DND-41, HPB-ALL and HEK293T cell lines were cultured as described in the *Online Supplementary Methods*.

In vitro *CK2 kinase assay*

CK2 activity was measured using the Casein Kinase 2 Assay Kit (Millipore) according to the manufacturer's instructions, as previously described.[19] Kinase activity was calculated by subtracting the substrate-less background for each sample

Transfection of HEK293T cells

Vectors bearing human JAK3, γC, IL-7Rα, and mouse Stat5a were used to reconstitute the IL-7 signaling machinery in

Table 1. Immunophenotypic characteristics, maturation stage and *IL7R* mutational status of T-ALL primary cells and cell lines.

	CD1a	CD2	CD3	cCD3	CD5	CD7	CD4	CD8	Maturation stage	*IL7R* mutation
Primary Samples										
T-ALL#1	+	–	–	+	+	+	ND	ND	III (cortical)	–
T-ALL#2	–	+	ND	+	-	ND	–	–	II or IV (non-cortical)	–
T-ALL#3	ND	–	–	+	+	+	–	–	II (pre-T)	ND
T-ALL#4	+	+	–	+	+	+	+	+	III (cortical)	–
T-ALL#5	ND	+	–	+	+	+	–	–	II (pre-T) or III (cortical)	–
T-ALL#6	+	+	–	ND	+	+	–	–	III (cortical)	–
T-ALL#7	–	+	–	+	+	+	–	+	II (pre-T)	–
T-ALL#8	+	+	+	ND	+	+	+	ND	III (cortical)	+
Cell Lines										
TAIL7	–	+	–	+	+	+	+	+	II (pre-T)	–
DND-41	+	+	+	+	+	+	+	–	III (cortical)	+
HPB-ALL	+	+	+	+	+	+	+	+	III (cortical)	–

ND: not determined; TALL: T-cell acute lymphoblastic leukemia; +: positive; -:negative.

HEK293T cells (which express only JAK1 endogenously). Vectors bearing the CK2α and α' subunit were kindly provided by D.W. Litchfield.[32] Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Transfected cells were stimulated with IL-7 (100ng/ml) for 15 minutes and 6 hours at 37°C. Reactions were stopped by placing samples on ice.

Immunoblotting

Lysates were prepared as described,[33] resolved by SDS-PAGE, and immunoblotted with antibodies against p-JAK3 (Y980), JAK3, JAK1, STAT5, Cyclin A, Cyclin E, Cyclin D2, CK2α, CK2α', CK2β, Actin (Santa Cruz Biotechnology, Inc.), p-STAT5a/b (Y694/Y699) (Millipore), p-JAK1 (Y1022/1023), p-Akt (S473), p-PTEN (S380), Akt, PTEN (Cell Signaling Technology), $p27^{Kip1}$ (BD Biosciences), PARP (Novus Biologicals) and p-Akt (S129) (Abgent). Densitometry analysis was performed using Adobe Photoshop CS5 Extended software (Adobe Systems). Results were normalized to the loading control.

Analysis of cell growth, activation and viability

Cell growth was determined as described.[34] The activation marker CD71 was measured using FITC-conjugated anti-CD71 (eBioscience) antibody. Results were expressed as the percentage

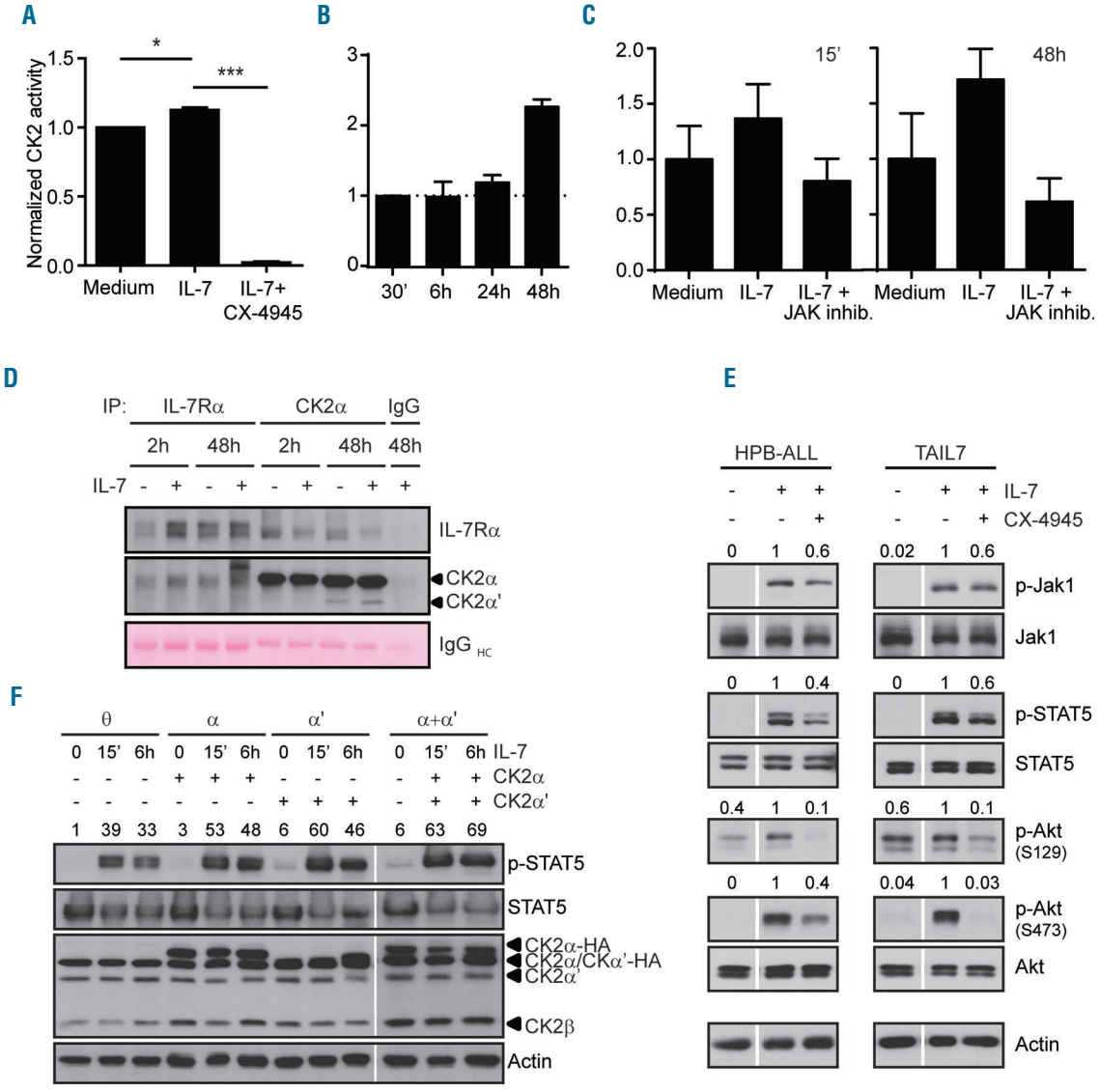


Figure 1. CK2 activity modulates IL-7-mediated signaling in T-ALL cells. (A) TAIL7 cells were pre-incubated with the CK2-specific inhibitor CX-4945 (6μM) for 2 hours and stimulated with IL-7 (50ng/ml) for 15 minutes. Data represent normalized mean±sem from two independent experiments. *P<0.05, ***P<0.001 (One-way ANOVA, with Tukey's post-test). (B) TAIL7 cells were incubated with IL7 for the indicated time periods. (C) TAIL7 cells were pre-incubated with a pan-Jak inhibitor (10μM) for 2 hours and stimulated with IL-7 for 15 minutes or 48 hours. CK2 activity was determined as described in 'Methods'. (D) TAIL7 cells were incubated with or without IL-7 for the indicated time points. Immunoprecipitation of IL-7Rα, CK2α and IgG (negative control) was followed by immunoblotting with anti-IL-7Rα and anti-CK2α antibodies. Ponceau staining of IgG heavy chain was used as loading control. (E) TAIL7 and HPB-ALL cells were pre-incubated for 2 hours with CX-4945 and stimulated with IL-7 for 15 minutes. Total protein extracts were resolved by SDS-PAGE, and total and phosphorylated proteins were detected by immunoblot. (F) HEK293T cells were transfected with CK2α and/or CK2α' subunits or mock control (θ) and stimulated with IL-7 (100ng/ml) for 15 minutes. (E,F) Values correspond to load control-normalized densitometric ratios of phospho-proteins. CK2: casein kinase 2; IL-7: interleukin 7; IgG: immunoglobulin G; p-Jak1: phospho-janus kinase 1; p-STAT5: phospho-signal transducer and activator of transcription 5; p-Akt: phospho-protein kinase B; T-ALL: T-cell acute lymphoblastic leukemia; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

of positive cells and as the specific mean intensity of fluorescence (MIF).[34] The determination of cell viability was performed by flow cytometry analysis of FSCxSSC distribution, and by Annexin V (eBioscience) and 7-AAD (BD Biosciences) staining.

Proliferation assays

Proliferation was assessed as described.[5]

Intracellular staining

Bcl-2 expression was determined by intracellular staining using a cell permeabilization kit according to the manufacturer's instructions (ADG Bio Research GmbH), and detected by flow cytometry with FITC-conjugated anti–Bcl-2 antibody (Dako).

Assessment of mitochondrial membrane potential ($\Delta\psi m$)

Cells were harvested, stained in RPMI1640 medium with TMRE (Sigma-Aldrich) to a final concentration of 100nM, incubated for 15 minutes at 37°C with 5% CO2, and analyzed by flow cytometry.

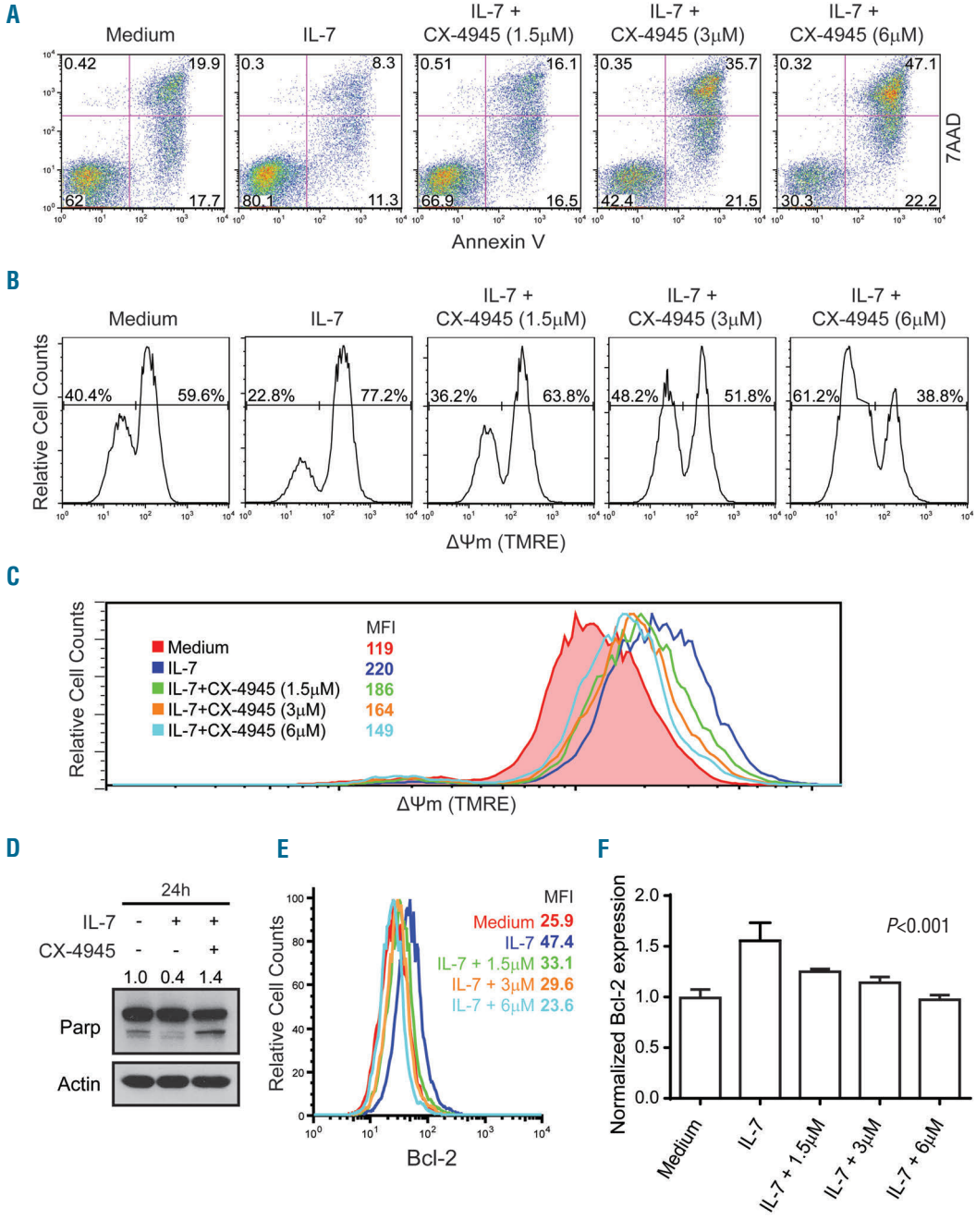


Figure 2. CK2 activity is required for IL-7-mediated Bcl-2 upregulation, mitochondrial homeostasis and prevention of T-ALL cell apoptosis. TAIL7 cells were cultured with IL-7 (10ng/ml) in the presence of increasing concentrations of the CK2 inhibitor CX-4945, as indicated, and analyzed for (A) apoptosis at 96 hours; (B,C) mitochondrial membrane potential (ΔΨm) at 72 hours, gated in the whole population (B) as an additional measure of overall apoptosis, or within the live cell population (C) as a measure of a very early event in apoptosis; and (D) PARP cleavage at 24 hours. (E,F) Bcl-2 levels were analyzed by flow cytometry at 72 hours. Mean fluorescence intensity (MFI) are indicated for each condition. Data are representative (E) of the mean±sem (F) of two independent experiments. CK2: casein kinase 2; IL-7: interleukin 7; Bcl-2: B-cell lymphoma 2; T-ALL: T-cell acute lymphoblastic leukemia; PARP: poly ADP (Adenosine, Diphosphate)-ribose polymerase; TMRE: tetramethylrhodamine ethyl ester.

Cell cycle analysis

DNA content after staining with propidium iodide was measured as described previously.[5] Cell cycle distribution was determined using ModFit LT software (Verity Software House).

Statistical analysis

FlowJo Software (Tree Star Inc.) was used to analyze flow cytometry data. GraphPad Prism software was used for statistical analysis. Differences between mean values were calculated using two-tailed Student's *t*-test and two-way ANOVA, as appropriate. $P<0.05$ was considered significant.

Additional methods are available in the *Online Supplementary Methods*.

Results

Optimal IL-7-mediated signaling requires CK2 activity in T-ALL cells

We previously showed that CK2 is overexpressed and hyperactivated in T-ALL[23] (*Online Supplementary Figure S1A*), and that T-ALL cells can benefit from IL-7 *in vitro*[5,34] and *in vivo*.[11] We now questioned whether a link could exist between IL-7-mediated signaling and CK2 activity. We determined CK2 activity in human IL-7-dependent TAIL7 T-ALL cells, which display the biological and signaling properties of primary leukemia cells.[31] IL-7 induced rapid (within 15 minutes) yet very mild

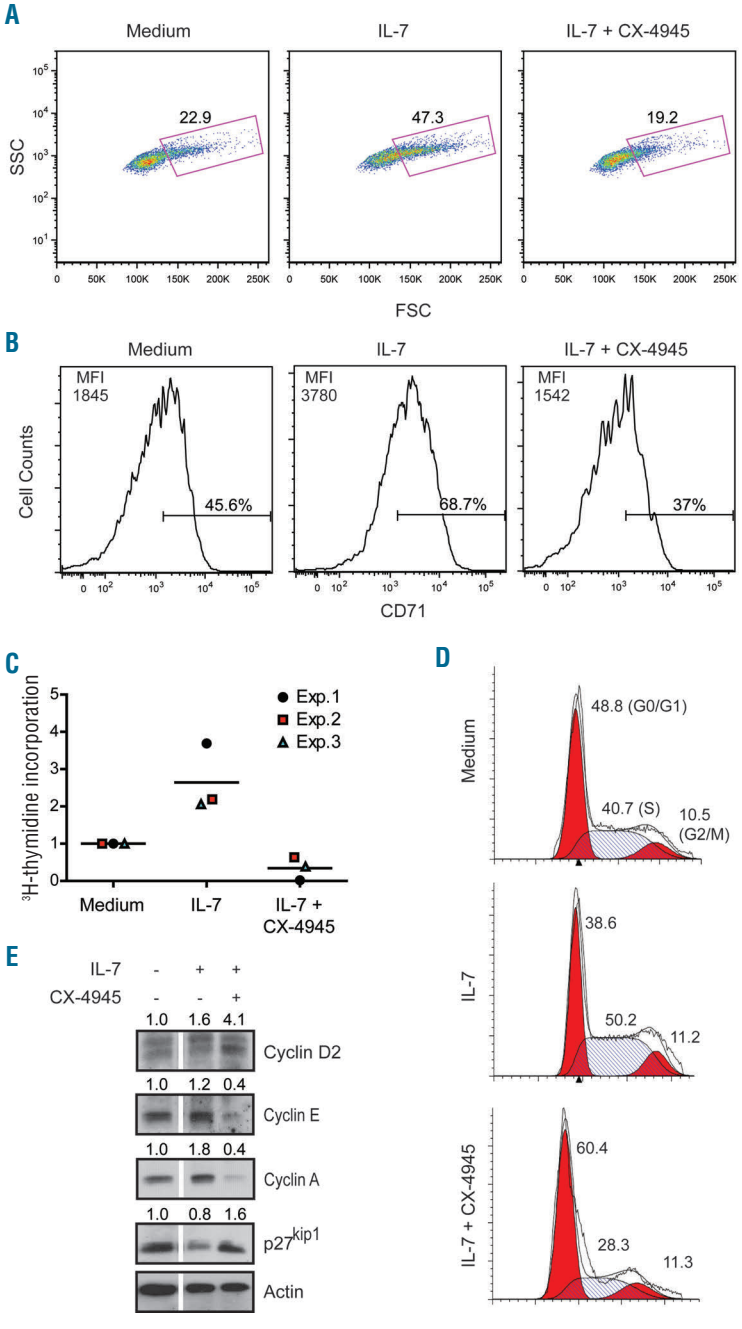


Figure 3. CK2 activity is essential for IL-7-mediated T-ALL cell growth and cell cycle progression. TAIL7 and HPB-ALL cells were cultured in medium alone or with IL-7 (20ng/ml), in the presence or absence of CX-4945 (6μM), and analyzed at the time points indicated below. Results were similar for both cell lines and representative data are presented. (A) TAIL7 cell size increase (cell growth) was determined by analysis of FSC distribution by flow cytometry after 72 hours. (B) Expression of the 'activation' marker CD71 (transferring receptor) at the surface of HPB-ALL cells was measured by flow cytometry at 72 hours. (C) Proliferation of TAIL7 cells was assessed at 72 hours by ^{3}H-thymidine incorporation as described in 'Methods'. (D) Cell cycle profile of TAIL7 cells was assessed by PI staining of fixed cells and analyzed by flow cytometry at 48 hours. (E) The corresponding expression profile of the indicated cell cycle regulators was measured by immunoblot. Values denote load control-normalized densitometric ratios. Results are representative of 2 to 3 independent experiments. CK2: casein kinase 2; IL-7: interleukin 7; T-ALL: T-cell acute lymphoblastic leukemia; PI: propidium iodide; MFI: mean fluorescence intensity.

upregulation of CK2 activity, which was completely inhibited by pre-treatment with CX-4945, a clinical-grade, highly selective CK2 inhibitor[35] (Figure 1A). This transient early response, which was no longer detectable 30 minutes after IL-7 stimulation, was followed by late, more robust increased CK2 activation at 24-48 hours (Figure 1B). IL-7 did not significantly alter the expression of the transcript (*Online Supplementary Figure S1B*) or protein (*Online Supplementary Figure S1C*) levels of any of the CK2 isoforms (α, α' or β), suggesting that IL-7-mediated upregulation of CK2 activity was independent of the regulation of CK2 expression levels.

JAK1 and JAK3 associate with IL-7Rα and the other IL-7R subunit (γc), respectively.[36] The blockade of IL-7 signaling using a pan-JAK inhibitor (Pyridone 6) abrogated CK2 activity upon short- and long-term IL-7 stimulation

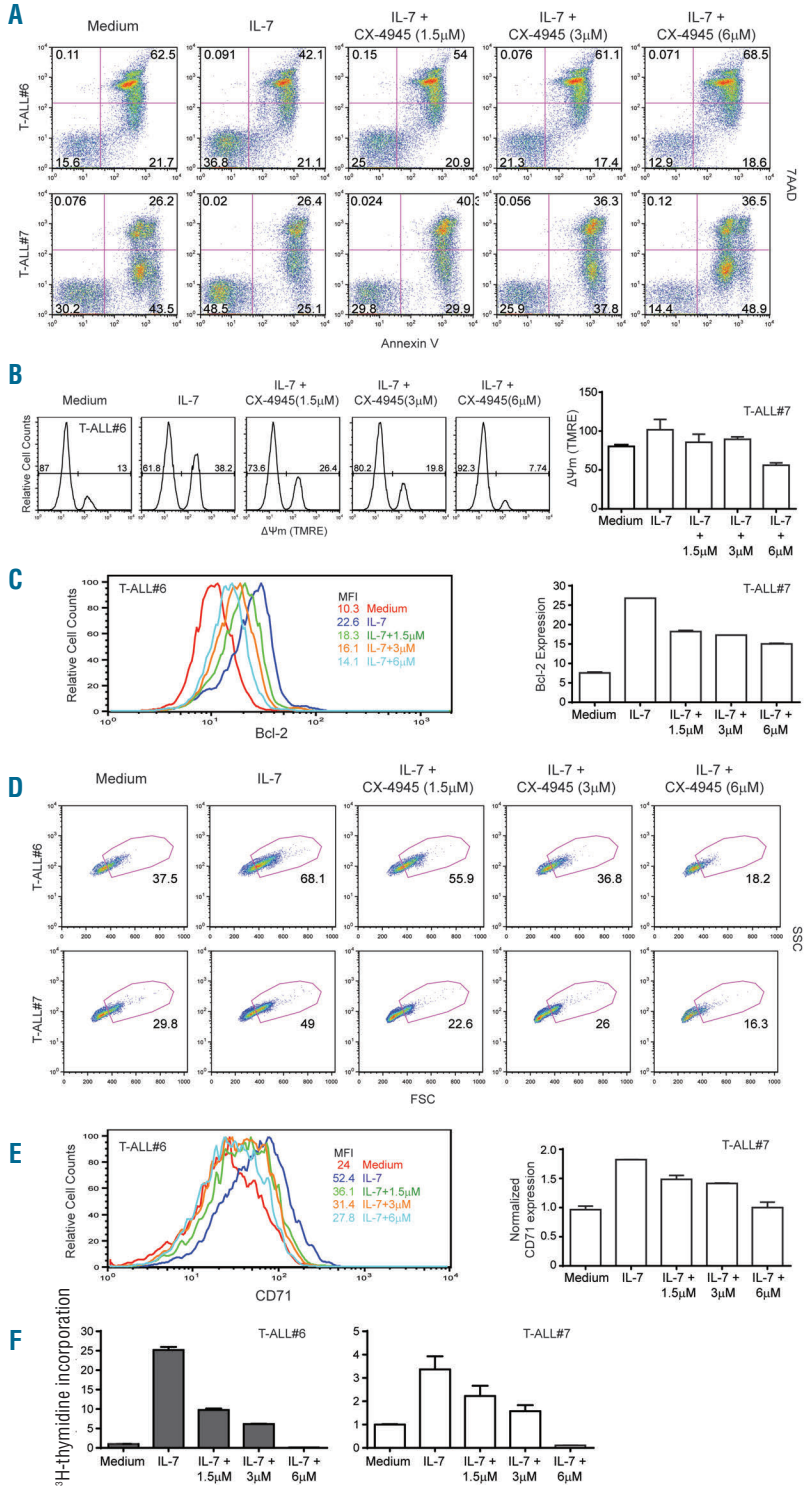


Figure 4. CK2 inhibition abolishes IL-7-mediated viability, activation and proliferation of primary T-ALL blasts from diagnostic pediatric patients. Primary T-ALL cells were cultured for 24 hours (T-ALL#6) or 48 hours (T-ALL#7) in medium alone or with IL-7 (20ng/ml) in the presence of increasing concentrations of CX-4945, as indicated. Viability and apoptosis were assessed by Annexin V/7-AAD staining (A), TMRE (B) and Bcl-2 expression (C). 'Activation' status was assessed by cell size determination (D) and CD71 surface expression (E). Proliferation was determined by ^{3}H-thymidine incorporation at 72 hours (F) as described in 'Methods'. CK2: casein kinase 2; IL-7: interleukin 7; T-ALL: T-cell acute lymphoblastic leukemia; TMRE: tetramethylrhodamine ethyl ester; Bcl-2: B-cell lymphoma 2; MFI: mean fluorescence intensity.

(Figure 1C), indicating that JAKs are required for IL-7R-mediated CK2 activation in T-ALL cells. To further characterize the mechanisms linking IL-7 and CK2, we performed co-immunoprecipitation experiments involving IL-7Rα and CK2α. We found that IL-7Rα co-immunoprecipitated CK2α and vice-versa in both non-stimulated and IL-7-treated TAIL7 cells (Figure 1D). This indicates that IL-7Rα and CK2α physically interact at steady-state, and

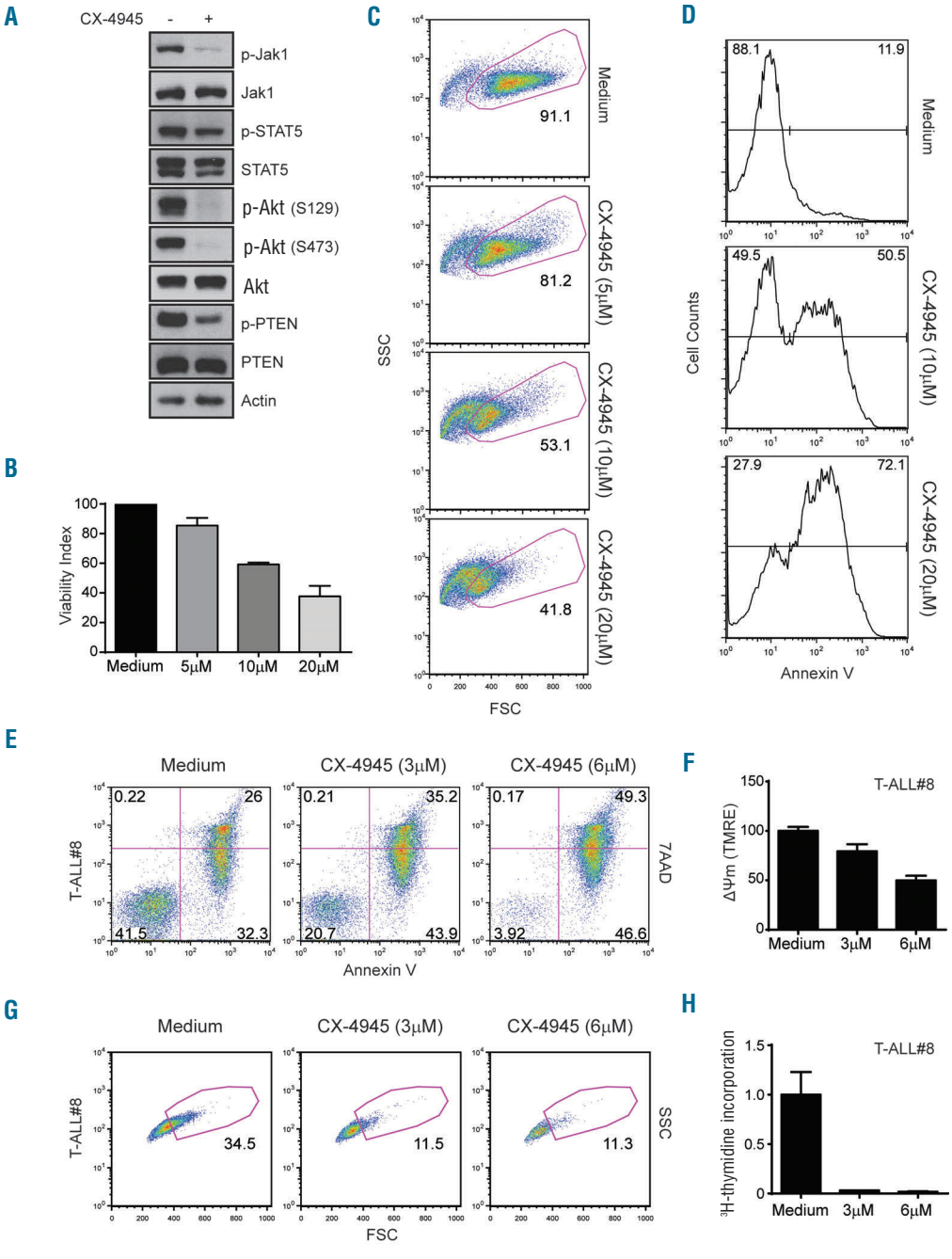


Figure 5. CK2 inhibition abolishes constitutive IL-7R-mediated signaling and survival of T-ALL cells expressing mutant IL-7Rα. (A) DND-41 cells were cultured for 24 hours in the presence or absence of CX-4945 (10μM). Lysates were resolved by SDS-PAGE and analyzed for the levels of phosphorylation of the indicated proteins. Total Akt was used as loading control. (B-D) DND-41 cells were cultured in medium alone or in the presence of the indicated doses of CX-4945, and their viability was analyzed at 48 hours by FSCxSSC flow cytometry discrimination (B, C) and Annexin V staining (D). Results in (B) are summarized as mean±sem of four independent experiments. Data in (A-D) are representative of 4 independent experiments. (E-H). Primary mutant IL7R (p.Leu242_Leu243insAsnProCys) leukemia cells from patient #8 (see Table 1) were cultured in medium alone or in the presence of the indicated doses of CX-4945. (E) Viability was assessed by Annexin V/ 7-AAD staining at 72 hours. (F) Mitochondrial membrane potential (ΔΨm) was evaluated at 48 hours after TMRE staining, gated on the live population. (G) Cell growth was determined by analysis of FSC distribution by flow cytometry at 72 hours. (H) Proliferation was determined by ^{3}H-thymidine incorporation. Results in (F) and (H) are summarized as mean±sem of 2 replicate analyses. CK2: casein kinase 2; IL-7: interleukin 7; T-ALL: T-cell acute lymphoblastic leukemia; TMRE: tetramethylrhodamine ethyl ester; p-Jak1: phospho-janus kinase 1; p-STAT5: phospho-signal transducer and activator of transcription 5; p-Akt: phospho-protein kinase B/murine thymoma viral oncogene homolog 1; (p-)PTEN: (phospho)-phosphatase and tensin homolog deleted on chromosome ten; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

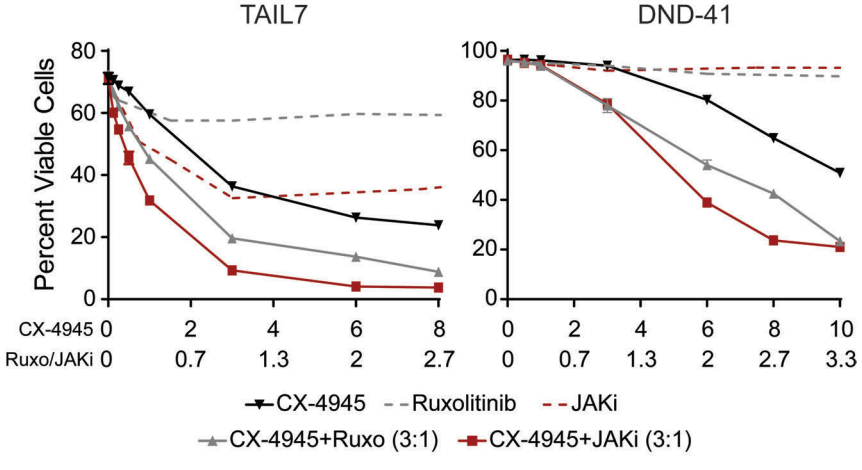
that this interaction is maintained after IL-7 stimulation.

Next, we assessed whether CK2 activity could impact on IL-7-mediated signaling in T-ALL. We pre-treated TAIL7 and HPB-ALL (IL-7-responsive) T-ALL cells with CX-4945 for 2 hours, stimulated each cell line with IL-7 for 15 minutes, and determined the phosphorylation status of known IL-7-activated signaling pathways (Figure 1E). Akt is phosphorylated at S129 by CK2.[37] In accordance with the CK2 kinase activity assay, IL-7 promoted only a minor increase in Akt phospho-S129 levels, which were strongly downregulated by CX-4945. IL-7-dependent activation of the JAK/STAT pathway, measured by the increase in phospho-STAT5 and phospho-JAK1 levels, was significantly downregulated upon abrogation of CK2 activity (Figure 1E and *Online Supplementary Figure S2*). Likewise, IL-7-induced PI3K/Akt pathway activation was significantly prevented by CK2 inhibition, as determined by the levels of Akt S473 phosphorylation (Figure 1E and *Online Supplementary Figure S2*). To further confirm the relevance of CK2 for optimal IL-7-mediated signaling, we reconstituted all the elements of the IL-7 receptor signaling machinery in HEK293T cells,[13] in the presence or absence of forced expression of CK2α and/or α' subunits. We found that overexpression of CK2 significantly augmented IL-7-mediated signaling as evaluated by STAT5 phosphorylation (Figure 1F). Overall, these experiments indicate that CK2 activity is essential for maximal IL-7/IL-7R-mediated signaling, impacting both PI3K/Akt and JAK/STAT pathways.

CK2 activity is essential for IL-7-mediated T-ALL cell viability

IL-7 has the ability to promote leukemic T-cell viability and proliferation through PI3K/Akt[34] and JAK/STAT5 pathways.[38] Therefore, we next sought to evaluate the consequences of CK2 inhibition on the functional outcomes of IL-7 upon T-ALL cells. The culture of TAIL7 or HPB-ALL cells in the presence of IL-7 with or without concomitant treatment with CX-4945, demonstrated that IL-7-mediated upregulation of leukemia cell viability was completely prevented by CK2 inhibition (Figure 2A, *Online Supplementary Figures S3 and S4A*). This effect was dose-dependent (Figure 2), and mainly due to increased apoptosis, as shown by 7-AAD and Annexin V staining (Figure 2A and *Online Supplementary Figure S4A*), mitochondrial transmembrane potential (Figure 2B,C and *Online Supplementary Figures S4B and S4C*) and PARP cleavage (Figure 2D). In agreement, IL-7-mediated upregulation of Bcl-2, which is mandatory for the pro-survival effects of IL-7,[5,34,39] was significantly abrogated by CX-4945 (Figure

A



B

C

TAIL7 Drug	Combination Index (Ci) ED50	Dm (potency) µM
CX-4945	-	3.9
CX-4945+Ruxo (3:1)	0.35	1.4
CX-4945+JAKi (3:1)	0.17	0.7

D

DND-41 Drug	Combination Index (Ci) ED50	Dm (potency) µM
CX-4945	-	10.5
CX-4945+Ruxo (3:1)	0.61	6.4
CX-4945+JAKi (3:1)	0.49	5.2

Figure 6. CK2 and JAK inhibitors synergize in inducing cell death of IL-7R mutant-expressing and IL-7-stimulated T-ALL cells. TAIL7 (A,C) and DND-41 (B,D) cells were cultured in the presence of increasing amounts of CX-4945 alone or combined with pan-JAK inhibitor (JAKi) or JAK1/2 inhibitor Ruxolitinib (Ruxo) for 72 hours. (A,B) Viability was determined by FSCxSSC discrimination. (C,D) Synergistic effect determination, combination index (CI) and median-effect dose (Dm) calculation was performed as described in the *Online Supplementary Methods*. CK2: casein kinase 2; IL-7: interleukin 7; IL-7R: interleukin 7 receptor; T-ALL: T-cell acute lymphoblastic leukemia.

2E,F and *Online Supplementary Figure S4D*). These results, which were corroborated using the unrelated CK2 small molecule inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB)[23] (*Online Supplementary Figure S5*), strongly suggest that CK2 activity is required for IL-7-mediated viability of T-ALL cells.

CK2 activity is essential for IL-7-mediated T-ALL cell growth and cell cycle progression

IL-7 promotes hypertrophy of T-ALL cells, which is associated with augmented metabolism as measured by increased glucose uptake.[5,34] To understand the effect of CK2 on IL-7-mediated leukemia cell growth, we incubated TAIL7 cells with IL-7 and CX-4945 or TBB, and determined cell size by FSCxSSC flow cytometry discrimination, where bigger cells (higher FSC) tend to be more metabolically active and proliferating. Our analyses demonstrate that the increase in cell size triggered by IL-7 was abolished by co-treatment with CX-4945 (Figure 3A) in a dose-dependent manner (*Online Supplementary Figure S6A*). Likewise, TBB prevented IL-7-mediated T-ALL cell growth (*Online Supplementary Figure S7*). Moreover, IL-7-dependent surface upregulation of the transferrin receptor (CD71), which associates with T-ALL cell growth,[34] was reversed by CK2 inhibition (Figure 3B and *Online Supplementary Figure S6B*).

Because growth of lymphoid cells is often associated with cell division, we next evaluated the impact of CK2 activity on IL-7-dependent T-ALL cell proliferation. In agreement with our previous reports,[11,31,34] IL-7 increased ^{3}H-thymidine incorporation, indicative of cell cycle progression into S-phase. This effect was reversed in the presence of CX-4945 (Figure 3C and *Online Supplementary Figure S6C)* and TBB (*Online Supplementary Figure S8*). In agreement with these data, analysis of the cell cycle profile of TAIL7 cells demonstrated that IL-7 led to an increase in the frequency of cells in S-phase, which was completely abolished by CK2 inhibition (Figure 3D and *Online Supplementary Figure S9*). At the molecular level, IL-7 promoted an increase in the expression of cyclins A and E, which are involved in S-phase entry and progression towards G2/M, and a decrease in the cyclin-dependent kinase p27^{Kip1}, whose expression contributes to prevent cell cycle progression past G1. In agreement with progression towards S-phase, the G1-associated cyclin D2 was mildly downregulated by IL-7. Upon inhibition of CK2 activity with CX-4945, these effects were completely reversed (Figure 3E), in accordance with the accumulation of TAIL7 cells in G1 (Figure 3D). These data demonstrate that CK2 kinase activity is required for IL-7 to promote growth and cell cycle progression of T-ALL cells.

CK2 activity is mandatory for IL-7-mediated viability, growth and proliferation of primary T-ALL cells from diagnostic patients

Because T-ALL cell lines may accumulate alterations that do not necessarily reflect primary disease, we next sought to extend the pathophysiological relevance of our

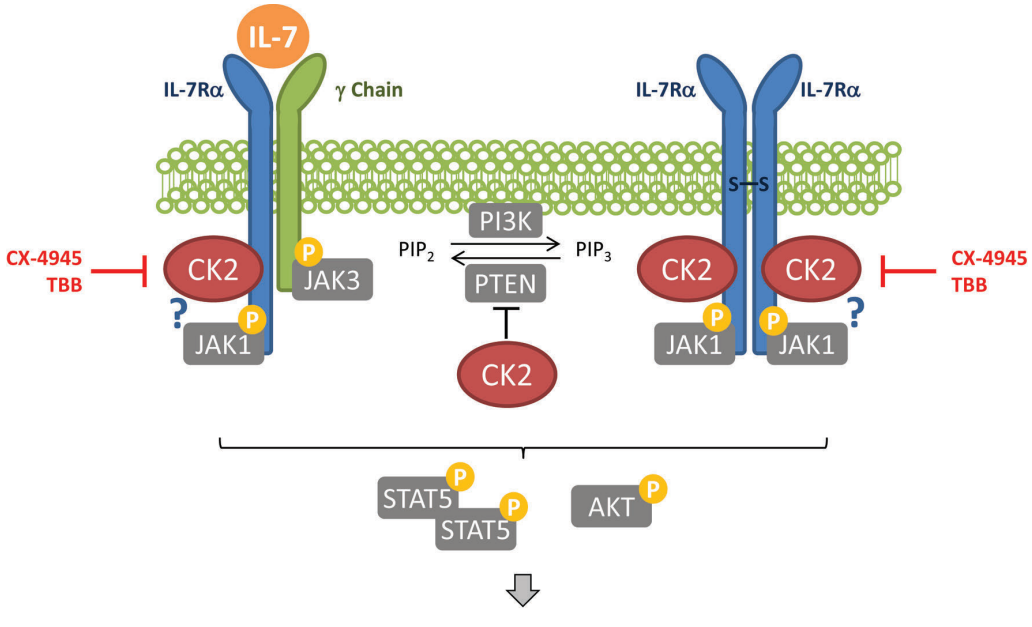


Figure 7. Model for CK2 involvement in IL-7/IL-7R-mediated signaling in T-ALL. In addition to its known role on PTEN posttranslational inactivation in T-ALL, CK2 appears to bind to the IL-7Rα chain and to be required for optimal IL-7R-mediated signaling, both in response to IL-7 and as a result of mutational activation of the receptor. Although the exact mechanisms by which CK2 may be regulated by and impact on JAK activity require further research, the consequence is that the activation of IL-7R downstream effectors, namely STAT5 and Akt, relies on CK2 activity. Thus, the use of CK2 inhibitors (such as CX-4945 or TBB) may be therapeutically relevant not only because they block CK2-mediated PTEN inactivation but also, as reported herein, because they are able to abrogate IL-7/IL-7R-mediated pro-leukemia signaling. CK2: casein kinase 2; IL-7: interleukin 7; IL-7R: interleukin 7 receptor; T-ALL: T-cell acute lymphoblastic leukemia; PTEN: phosphatase and tensin homolog deleted on chromosome ten; TBB: 4,5,6,7-tetrabromobenzotriazole; PIP2: phosphatidylinositol 4,5-bisphosphate; PIP3: phosphatidylinositol 3,4,5-trisphosphate; PI3K: phosphoinositide 3-kinase.

findings by determining whether these were reproduced in T-ALL blasts collected from patients at diagnosis. We confirmed that IL-7 had a major positive impact on the viability of all the primary T-ALL patient samples we analyzed, with significant downregulation of spontaneous apoptosis. This effect was completely reversed by CK2 inhibition in all cases (n=8; Figure 4A, *Online Supplementary Figure S10A* and *data not shown*). Analysis of mitochondrial transmembrane potential confirmed that CK2 modulates the ability of IL-7 to prevent this very early sign of apoptosis (Figure 4B and *Online Supplementary Figure S10B*). This process, similar to that which was observed in TAIL7 and HPB-ALL cells, likely reflects IL-7-triggered fluctuations in Bcl-2 expression that are dependent on CK2 activity (Figure 4C and *Online Supplementary Figure S10C*). The similarities between T-ALL cell lines and primary leukemia cells extended beyond the impact on cell survival. Upon CK2 inhibition T-ALL blasts were no longer able to augment their size (Figure 4D, *Online Supplementary Figures S7 and S10D*) or CD71 surface expression (Figure 4E and *Online Supplementary Figure S10E*) in the presence of IL-7. Accordingly, the well known proliferative effect of IL-7 on primary T-ALL cells[9] was blocked by CX-4549 (Figure 4F and *Online Supplementary Figure S10F*) in a dose-dependent fashion (Figure 4F). Likewise, TBB prevented IL-7-mediated proliferation of primary T-ALL cells (Online *Supplementary Figure S8*). These data taken together indicate that CK2 is a key element in IL-7-mediated promotion of viability, growth and proliferation of T-ALL blasts.

CK2 inhibition abrogates constitutive signaling and viability of mutant IL7R*-expressing T-ALL cells*

We and others have shown that around 10% of pediatric T-ALL cases display gain-of-function mutations in the α chain of the IL-7R, leading to constitutive activation of downstream signaling, namely PI3K/Akt and STAT5, with the consequent promotion of cell proliferation, transformation[12,13,40] and tumorigenesis.[13,14,16] Therefore, we next sought to determine whether CK2 is also required in the context of the signals elicited by mutant *IL7R*. We found that CK2 inhibition prevented constitutive signaling downstream from mutated *IL7R* in DND-41 T-ALL cells,[16] as determined by the levels of phosphorylation of Akt and STAT5 (Figure 5A). Accordingly, CX-4945 induced DND-41 cell death in a time- and dose-dependent manner (Figure 5B,C), which was associated with high levels of apoptosis (Figure 5D).

Next, we extended our analysis to a primary T-ALL diagnostic sample (T-ALL#8, Table 1) displaying *IL7R* mutational activation, as previously characterized (patient P1 in reference 13). Remarkably, the CK2 inhibitor promoted apoptosis (Figure 5E,F) and atrophy (Figure 5G), and prevented proliferation (Figure 5H) of leukemia blasts at concentrations even lower than those required for DND-41 cells. Overall, these results indicate that, similar to T-ALL cells stimulated with IL-7, cells displaying mutant *IL7R* remain sensitive to abrogation of CK2 activity.

Combined CK2 and JAK inhibition is synergistic against both IL-7-dependent and mutant IL7R*-expressing T-ALL cells*

To further generate preliminary evidence of the clinical potential of our observations we next investigated whether the combination of CX-4945 with JAK inhibitors would result in more efficient elimination of IL-7/IL-7R-mediated T-ALL cell viability. Treatment of TAIL7 cells with a combination of CX-4945 and either the pan-JAK inhibitor or the JAK1/2 clinical-stage inhibitor ruxolitinib, synergized in preventing IL-7-mediated viability (Figure 6A and 6C). The same combinations also displayed a synergistic effect in inducing cell death of mutant IL-7R-expressing DND-41 cells (Figure 6B and 6D). These results suggest that inhibiting concomitantly CK2 and JAK may be particularly effective in targeting both IL-7- and mutant *IL7R*-dependent T-ALL cells.

Discussion

Throughout the years, considerable evidence has accumulated pinpointing the importance of the IL-7/IL-7R axis for T-cell leukemogenesis.[26] The pro-oncogenic role of IL-7 and IL-7R in human T-ALL has been clearly highlighted by recent data, revealing that IL-7 significantly accelerates T-ALL disease progression *in vivo*[11] and that gain-of-function mutations in the IL-7R exist in T-ALL patients, including in poor prognosis cases.[12,13,40] On the other hand, CK2 is frequently overexpressed and hyperactivated in T-ALL,[23] driving PI3K/Akt pathway activation by posttranslationally inhibiting PTEN.[23] In the present studies we sought to determine whether CK2 and IL-7/IL-7R could be functionally linked, by evaluating whether CK2 is involved in the mechanisms underlying IL-7/IL-7R-mediated effects in T-ALL cells.

We demonstrated that IL-7 upregulates CK2 activity in a minor but significant manner, with clear biological impacts. The underlying mechanisms remain to be elucidated. CK2 has been shown to bind to JAK kinases,[25] which in turn are known to associate with the IL-7R. In agreement, we have demonstrated that CK2 interacts with IL-7Rα. Thus, it is possible that CK2 activity is upregulated in the context of multimeric complexes involving the IL-7 receptor and JAK1. Consistent with this was the demonstration that JAK inhibition impeded IL-7-mediated CK2 activation. Given that Akt was recently shown to phosphorylate and thereby regulate CK2,[41] it is also possible that CK2 may be involved in complex loops in which it is both regulated by and a regulator of PI3K/Akt signaling downstream from IL-7/IL-7R. This has a precedent in mTOR, which is activated downstream of Akt within the mTORC1 complex and is responsible for Akt activation as part of mTORC2.[42] Interestingly, mTOR is also an important component of the IL-7 signaling network in ALL.[5,43,44] These considerations apart, we found that CK2 activity is absolutely required for maximal IL-7-mediated signaling. This was demonstrated by using two distinct CK2-specific pharmacological inhibitors, TBB and CX-4945 (Silmitasertib), the latter of which has entered phase I clinical trials for refractory solid tumors and multiple myeloma,[45] and is a well-characterized, highly specific inhibitor of CK2.[35] We also tried silencing the expression of CK2α and β subunits. Notably, although we were able to efficiently knockdown CK2 in T-ALL cells by up to 80%, we only partially eliminated CK2 activity, which was sufficient to maintain normal levels of Akt S129 phosphorylation and the viability of T-ALL cells (*data not shown*). Moreover, CRISPR/Cas9-mediated deletion of CK2 failed to produce viable T-ALL cells, suggesting that minimal CK2 expression is sufficient to maintain biologi-

cally relevant kinase activity that is absolutely required for T-ALL cell viability. Thus, we used the opposite strategy and forced CK2α and/or CK2α' expression in HEK293T cells ectopically expressing the IL-7 receptor signaling machinery, thereby demonstrating that CK2 overexpression augments IL-7/IL-7R-mediated STAT5 phosphorylation. This indicates that CK2 is effectively involved in IL-7-mediated signaling. Moreover, using DND-41 T-ALL cells, which display a cysteine-introducing IL-7Rα mutation, we demonstrated that CK2 is also required for constitutive signaling downstream from mutationally activated IL7R.

We further characterized the extent to which CK2 impacts on IL-7/IL-7R-mediated effects on T-ALL cells. We found that CK2 activity is required for IL-7-induced viability, cell size increase and T-ALL cell cycle progression past G0/G1. In accordance, IL-7-mediated leukemia T-cell proliferation also depends on CK2. These results are consistent with the fact that CK2 regulates IL-7-triggered JAK/STAT pathway activation, which is essential for IL-7-mediated leukemogenesis in mice,[8] as well as PI3K/Akt signaling, which is fundamental for IL-7-mediated effects on human T-ALL cells.[26,34,44] Whether CK2 is involved in the regulation of other microenvironmental signals that promote T-ALL expansion remains to be explored. Our preliminary data demonstrate that IL-4-mediated T-ALL cell viability and proliferation[46] was prevented by CK2 inhibition (*Online Supplementary Figure S11*). These observations are consistent with the possibility that CK2 may have a broader role in regulating different extracellular (pro-leukemogenic) stimuli. Evidently, this needs to be seen in light of the fact that T-ALL cells also rely on high constitutive, cell-intrinsic activation of CK2.[23] Accordingly, CK2 pharmacological inhibition decreased viability and promoted apoptosis of T-ALL cells cultured in medium alone, as previously reported.[23] Importantly, in agreement with the requirement of CK2 for optimal IL-7-mediated signaling, IL-7 was not able to fully reverse this effect (*Online Supplementary Figures S3 and S12*).

CK2 inhibition also leads to clear cell death of the mutant *IL7R* T-ALL cell line DND-41. This appears to be a natural corollary from both of these pathways being constitutively activated downstream of mutant IL-7R,[12,13] and is in agreement with the fact that Ba/F3 cells stably expressing mutant IL-7Rα are sensitive to CK2 inhibition (*data not shown*). Of note, our observations indicate that clinical grade CK2 inhibitors, such as CX-4945 (Silmitasertib)[23,35] or CIGB-300, may constitute valid therapeutic tools against T-ALL patients displaying IL-7Rα mutations, including a significant fraction of very poor prognosis ETP-ALL cases.[40]

Interestingly, IL-7 has also been shown to activate both PI3K/Akt and STAT5 in normal T-cell precursors, which are implicated in thymocyte proliferation and differentiation.[47,48] Whether CK2 plays a role in normal T-cell development and homeostasis or, alternatively, this is a feature that is restricted to leukemia cells, in association with their high levels of CK2 expression, remains an open question. Curiously, CK2 was shown to be a key element in the process of receptor internalization through the formation of clathrin-coated pits,[49] and we previously demonstrated that IL-7 promotes IL-7R internalization in both normal and leukemia T-cells, which is required for optimal IL-7-mediated signaling.[50] Hence, it is possible that CK2 may act as an IL-7R internalization regulator, and by this upstream effect modulate IL-7-mediated signaling, which could explain the effects observed in JAK1 protein phosphorylation status after CK2 inhibition. This possibility warrants further investigation.

Overall, our present study contributes to a better understanding of the regulation of IL-7 and IL-7R-mediated signals, identifying CK2 as a critical modulator of IL-7 functional effects on T-ALL cells. There is an increasing recognition of the relevance of IL-7 and its receptor for T-cell leukemogenesis and leukemia maintenance, especially after the identification of *IL7R* as a *bona fide* T-cell oncogene,[11-13,40] and clear evidence that CK2 is critical for the viability of T-ALL cells.[23,35] Our data add to this knowledge by placing CK2 at the center point of both basal and IL-7R-dependent activation of pro-survival and proliferative pathways (Figure 7), and strongly supporting the rationale for the testing of CK2 inhibitors in the context of T-ALL.

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