Targeting cytokine and therapy induced PIM1 activation in T-cell acute lymphoblastic leukemia and lymphoma

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Key Points:

- CD127$^+$ T-ALL and T-LBL are sensitive to *in vivo* PIM inhibition
- Acute induction chemotherapy triggers *in vivo PIM1* activation in residual human T-ALL/T-LBL cell
- Combination of the PIM inhibitor PIM447 with induction chemotherapy improves leukemic survival in a PDX model of CD127$^+$ T-ALL

Abstract (max 250 words)

T-cell acute lymphoblastic leukemia and lymphoma (T-ALL/T-LBL) are aggressive hematological malignancies that are currently treated with high dose chemotherapy. Over the last years, the search towards novel and less toxic therapeutic strategies for T-ALL/T-LBL patients has largely focused on the identification of cell intrinsic properties of the tumor cell. However, non cell autonomous activation of specific oncogenic pathways might also offer opportunities that could be exploited at the therapeutic level. In line with this, we here show that endogenous IL7 can increase the expression of the oncogenic kinase PIM1 in CD127+ T-ALL/T-LBL, thereby rendering these tumor cells sensitive to in vivo PIM inhibition. In addition, using different CD127+ T-ALL/T-LBL xenograft models, we also reveal that residual tumor cells, which remain present after short-term in vivo chemotherapy, display consistent upregulation of PIM1 as compared to bulk non-treated tumor cells. Notably, this effect was transient as increased PIM1 levels were not observed in reestablished disease after abrogation of the initial chemotherapy. Furthermore, we uncover that this phenomenon is, at least in part, mediated by the ability of glucocorticoids to cause transcriptional upregulation of IL7RA in T-ALL/T-LBL PDX cells, ultimately resulting in non-cell autonomous PIM1 upregulation by endogenous IL7. Finally, we confirm in vivo that chemotherapy in combination with a pan-PIM inhibitor can improve leukemia survival in a PDX model of CD127+ T-ALL. Altogether, our work reveals that IL7 and glucocorticoids coordinately drive aberrant activation of PIM1 and suggests that IL7 responsive CD127+ T-ALL and T-LBL patients could benefit from PIM inhibition during induction chemotherapy.

Introduction

T-cell acute lymphoblastic leukemia (T-ALL) and T-cell acute lymphoblastic lymphoma (T-LBL) are aggressive hematological malignancies that arise from the abnormal activation of oncogenes and inactivation of tumor suppressor genes, followed by a differentiation arrest and uncontrolled clonal expansion of immature thymocytes[1].

PIM1 is a known JAK-STAT target gene that recently emerged as a therapeutic target for the treatment of T-ALL and T-LBL[2-7]. PIM1 activation in primary T-ALL/T-LBLs can occur through T-cell receptor driven translocations[5,6] or activating mutations targeting *IL7RA*, *JAK1*, *JAK3* or *STAT5B*[5,6]. Based on these cell-intrinsic mechanisms, the number of patients that might eventually benefit from PIM inhibitor therapy remains relatively low. However, recent work suggested that activation of JAK-STAT signaling in T-ALL could also be achieved through non-cell autonomous mechanisms, such as stimulation by interleukin-7 (IL7), a cytokine that is abundantly present in the leukemic micro-environment[8,9]. Notably, the ability of T-ALL cells to increase STAT5 phosphorylation upon IL7 stimulation solely depended on surface IL7R (CD127) expression and corresponded with *in vivo* responses of patient derived xenografts towards the JAK1/2 inhibitor ruxolitinib, irrespective of the presence of activating IL7R/JAK/STAT pathway mutations[9]. This cell extrinsic mechanism of JAK-STAT pathway activation, mediated by endogenous IL7, suggests that the fraction of T-ALL and T-LBL patients that might benefit from *in vivo* PIM inhibitor therapy could be more substantial than originally anticipated.

Current T-ALL and T-LBL treatment protocols consist of induction, consolidation, intensification) and maintenance therapy using a variety of different chemotherapeutic agents[10-12]. During induction, the bulk of leukemic cells are supposed to be eradicated by a combination of glucocorticoids, asparaginase and vincristine. However, in most cases, some level of minimal residual disease (MRD) remains present during or after induction and these MRD measurements are used to guide risk stratification and determine treatment intensity in T-AL/T-LBL. Currently, the molecular mechanisms by which these residual malignant cells escape the effects of chemotherapy remain poorly understood and no MRD specific therapeutic targets have been identified in human T-ALL/T-LBL so far.

Notably, previous research has shown that glucocorticoids, one of the core components of T-ALL/T-LBL induction therapy, can directly induce *Il7r* expression in murine T-cells through NR3C1-directed binding at an enhancer near the *Il7r* locus[13,14]. Also in the human context, mature $CD4^+$ and $CD8^+$ T-cells were shown to induce *IL7RA* expression upon glucocorticoid treatment[15] through a glucocorticoid response element located about 2000bp upstream of the *IL7RA* TATA box[16]. Given this, glucocorticoids could potentially drive therapy induced and non-cell autonomous activation of the JAK-STAT pathway eventually leading to downstream PIM1 activation. Given that hyperactivation of JAK-STAT signaling has recently emerged as a mechanism of glucocorticoid resitance[7-9], one could hypothesize that T-ALL/T-LBL cells which escape induction therapy could also be characterized by increased PIM1 levels.
Here, we investigated the therapeutic relevance of both cytokine and chemotherapy induced PIM1 activation in T-ALL and T-LBL.

Methods

Cell lines and patient samples

Cell lines were purchased from the DSMZ repository (Braunschweig, Germany) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 100 mg/mL kanamycin sulfate, and 2 mM L-glutamine (hereafter called 10% RPMI) at 37°C with 5% CO2. Primary T-ALL cells for establishing patient-derived xenograft (PDX) models were acquired by informed consent from the Department of Pediatric Hematology-Oncology at Ghent University Hospital and Leuven University Hospital. These primary T-ALL samples were assigned to a specific molecular genetic subclass based on real-time polymerase chain reaction of *SIL-TAL1*, *TLX1*/*TLX3* or *MLL* fusion transcripts, or fluorescence in situ hybridization analysis of the LMO2 locus.

Quantitative real-time polymerase chain reactions

Total RNA was isolated using the miRNeasy micro or mini kit with gDNA eliminator columns (Qiagen). The iScript cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA. In case RNA isolation yielded very low concentrations, the cDNA was first

amplified with the SsoAdvanced PreAmp Supermix kit (Bio-Rad) using a pool of primers for *IL7RA* (Fw 5'-CGCCAGGAAAAGGATGAAA-3', Rev 5'-ATACATTGCTGCCGGTTGG-3'), *CISH* (Fw 5'-AAAACTGGTGCAGCCCTTTGTA-3', Rev 5'-GCCACCAGACGGTTGATGAC-3'), *PIM1* (Fw 5'-CGAGCATGACGAAGAGATCAT-3', Rev 5'-TCGAAGGTTGGCCTATCTGA-3') and the reference genes *HPRT1* (Fw 5'-TGACACTGGCAAAACAATGCA-3', Rev 5'-GGTCCTTTTCACCAGCAAGCT-3'), *B2M* (Fw 5'-GCTGTCTCCATGTTTGATGTATCT-3', Rev 5'-TCTCTGCTCCCCACCTCTAAGT-3') and *TBP* (Fw 5'-CACGAACCACGGCACTGATT-3', Rev 5'-TTTTCTTGCTGCCAGTCTGGAC-3'). The quantitative real-time polymerase chain reactions were performed using the SsoAdvanced SYBR Green Supermix (Bio-Rad) and were run on the LightCycler480 (Roche, model LC480). Every sample was analyzed in duplicate and the gene expression was standardized against 3 housekeeping genes.

Phospho-STAT5 flowcytometry

PDX spleen cells were *ex vivo* stimulated for 30 minutes with 100 ng/ml human IL7 (Peprotech) in 10% RPMI, washed and subsequently extracellularly stained with a fixable viability dye (eBioscience, catalog # 65-0866-18) and human CD45-FITC (Miltenyi, #130-110-631) to gate for human leukemic blasts. The cells were then fixed and permeabilized with the FIX & PERM Cell Permeabilization Kit from Invitrogen (GAS-004), using the methanol fixation modification to maintain the phospho sites, as described in the standard Invitrogen protocol. Samples were stained for 30 minutes on room temperature with phospho-STAT5 (Tyr694) Monoclonal Antibody (SRBCZX), APC conjugated from eBioscience (Catalog #17-9010-42). After washing away excess antibody, cells were analyzed on the flowcytometer and data analysis was done using FlowJo software.

Cell cycle assay

One million PDX spleen cells were treated for 48h with 1µM PIM447 dihydrochloride (MedChem Express) in 10% RPMI. Cells were washed twice after 48h and resuspended in 300µl cold DPBS without calcium and magnesium and 700µl icecold 70% ethanol was dropwise added to the cells, while being vortexed. Samples were incubated for at least one hour at -20°C, after which they were washed twice and

resuspended in 500µl cold PBS. RNase A (Qiagen, 19101; 0.5 mg/ml final concentration) was added and the cells were incubated for 1 hour at 37°C. Afterwards propidium iodide (PI, Life Technologies; 40µg/ml final concentration) was added and the samples were measured on the flowcytometer. Data analysis was done using FlowJo software.

Apoptosis assay

PDX spleen cells were treated for 48h with 1µM PIM447 dihydrochloride (MedChem Express) in 10% RPMI. Apoptosis assay was performed with 500.000 cells per condition using the FITC AnnexinV apoptosis detection kit I (BD Biosciences). Cells were washed twice with PBS and resuspended in 1x Binding Buffer to a concentration of 1 million cells per ml. 100.000 cells were transferred to fresh eppendorfs and were either left unstained as a control or stained with 5 µl AnnexinV-FITC and/or 5µl PI. Cells were incubated for 15 minutes at room temperature, after which 200 µl additional 1x Binding Buffer was added. The samples were then measured on the flow cytometer, data analysis was done using FlowJo software.

***In vivo* treatment of xenografts with PIM inhibitors and chemotherapy**

For the collection of a stock of spleen and bone marrow cells from our T-ALL/T-LBL cohort, nonobese diabetic/severe combined immunodeficient γ (NSG) mice were injected tail vein at 6 weeks of age with 150µL phosphate-buffered saline containing 2.5 x 10^6 human T-ALL bone marrow or T-LBL pleural effusion cells. At regular time points, leukemia engraftment was monitored by human CD45 staining (CD45-FITC antibody; Miltenyi Biotec) in peripheral blood after red blood cell lysis using flowcytometry analysis. Upon establishment of disease, human leukemic cells were isolated from the spleen and retransplanted into secondary recipients. When mice were leukemic, animals were sacrificed and the spleen weight and %hCD45-positive leukemic blasts in bone marrow and spleen were determined by flowcytometry and viable cells were either immediately used for *ex vivo* experiments or stored in -150°C until further use.

For *in vivo* monotherapy treatment with the PIM inhibitors AZD1208 and PIM447 dihydrochloride, a secondary or tertiary xenograft was established for PDX 6. When

the cells were engrafted and the mice were randomly divided into 4 groups of 3 mice and treatment was started. Mice were treated with 100 mg/kg body weight PIM447 dihydrochloride or with 30 mg/kg body weight AZD1208 or with vehicle via oral gavage for 2 weeks (5 days on, 2 days off). PIM447 was formulated in 50 mM sodium acetate buffer pH 4 and AZD1208 was formulated in DMSO/PEG400/0.5% methylcellulose in a 10:45:45 ratio. Percentage human CD45-positive (%hCD45) cells in the peripheral blood was weekly measured. After treatment, animals were sacrificed and the spleen weight and %hCD45-positive leukemic blasts in bone marrow and spleen were determined by flowcytometry.

For combination therapy with PIM447 dihydrochloride and a chemotherapeutic cocktail tertiary xenograft injections were performed in a cohort of 20 NSG mice. Upon detection of human CD45 leukemic blasts in peripheral blood, mice were randomized in 4 groups and treated with vehicle, 80 mg PIM447/kg body weight via oral gavage, chemotherapeutics via i.p. or both PIM447 and chemotherapeutics at same concentrations used in the monotherapy groups. Dexamethasone (Aacidexam), vincristine and L-asparaginase were formulated in 100% phosphate-buffered saline. The treatment schedule for chemotherapeutics was as follows: the first day a combination of 100 IU oncospar, 10 mg/kg dexamethasone and 10µg vincristine was given per animal. Day 2 until day 5 only dexamethasone (10 mg/kg) was given. Mice were treated for three weeks (5 days on, 2 days off) and %hCD45 in peripheral blood was followed weekly. The first week both the chemotherapeutic cocktail and PIM447 were given, the next weeks only PIM447. After treatment we monitored survival of the mice by means of ‘humane ethical endpoints’, where mice were sacrificed when they suffered from more than 20% weight loss, or more than 90% leukemic blasts in the peripheral blood.

The ethical committee on animal welfare at Ghent University Hospital approved all animal experiments.

Statistical analysis

GraphPad Prism 7.0 (La Jolla, CA) was used for statistical analyses. The Mann-Whitney *U* test was used to analyze differences between subgroups. Data were considered statistically significant for *P* values less than .05.

Results

Cytokine induced PIM1 activation in CD127$^+$ T-ALL and T-LBL

Previous studies have shown that a subset of T-ALL patients are able to activate JAK-STAT signaling in response to exogenous IL7, regardless of the mutational status of IL7R pathway genes[8,9]. Although initially reported for early T-cell precursor ALL (ETP-ALL), the exact genetic features of T-ALL/T-LBL patients that show this IL7 responsiveness remain largely unknown.

Given this, we analyzed phosphorylated STAT5 (Tyr694, pSTAT5) levels upon IL7 stimulation in a series of genetically well-characterized patient-derived xenograft (PDX) samples obtained from 8 T-ALL and 4 T-LBL pediatric patient samples (**Supplementary Table 1, Supplementary Figure 1**). From these, 4 T-ALL and 2 T-LBL cases showed IL7 induced pSTAT5 induction, which was not the case for the remaining 6 T-ALL/T-LBL samples (**Figure 1A**, **Supplementary Figure 1**). IL7 responsive T-ALL/T-LBL samples covered a broad spectrum of molecular-genetic subtypes, including immature, TLX1$^+$, TLX3$^+$, NKX2.1$^+$, HOXA$^+$ and TAL/LMO$^+$, and were characterized by high *IL7RA* expression. In contrast, non-responders were NKX2.5$^+$ or TAL/LMO$^+$ and displayed low *IL7RA* levels (**Figure 1B**). Cooperative somatic mutations identified in these T-ALL/T-LBL patient samples are summarized in Supplementary Table 1.

As expected, IL7 stimulation resulted in *PIM1* upregulation in responsive T-ALL/T-LBL patient samples (**Figure 1C**), suggesting that CD127$^+$ T-ALL/T-LBLs, lacking *IL7RA*/*JAK*/*STAT* mutations, might also benefit from PIM inhibition. To test this, we performed *in vivo* evaluation of the PIM inhibitors PIM447 and AZD1208 using T-ALL PDX#6 (*TLX3*$^+$), which lacks cell-intrinsic genetic abnormalities targeting PIM1 or the IL7R pathway but has the ability to activate PIM1 upon IL7 stimulation (**Figure 1A**, **Supplementary Figure 1**). PIM inhibitor treatment (2 weeks; 5 days on – 2 days off, PIM447 (100 mg/kg) and AZD1208 (30 mg/kg) resulted in a significant reduction of the leukemic burden, as measured by the percentage of blasts in peripheral blood (**Figure 1D**) or spleen size (**Figure 1E**) and revealed that PIM447 was more effective *in vivo* as compared to AZD1208. The superior activity of PIM447, which is the only PIM inhibitor that is currently still in clinical trials for hematological malignancies[17,18], was also confirmed *ex vivo* on PDX#6 cells obtained from the spleen (**Figure 1F**). Finally,

we confirmed that *ex vivo* PIM447 treatment triggered a block in the G1 phase of the cell cycle with a concomitant increase in early and late apoptotic cells (**Supplementary Figure 2**).

Thus, cytokine induced PIM1 activation in CD127$^+$ T-ALL/T-LBL patient samples can be therapeutically targeted by the PIM inhibitor PIM447.

Simultaneous *PIM1* activation by combined cell intrinsic and extrinsic effects in human T-ALL/T-LBL

PIM1 overexpression in T-ALL/T-LBL can be caused by genomic translocations involving the T-cell receptor β or activating mutations targeting the IL7R/JAK/STAT signaling pathway. However, and as shown above, PIM1 can also become activated through non-cell autonomous stimulation by exogenous IL7 in CD127$^+$ T-ALL/T-LBL.

Here, we evaluated if these different mechanisms of PIM1 activation are mutually exclusive or not. Stimulation of HPB-ALL cells, which already express considerable amounts of PIM1 due to the presence of a JAK1 mutation, showed clear pSTAT5 induction which coincided with a significant upregulation of *PIM1* (**Figure 2A**). Similarly, IL7 stimulation of *ex vivo* treated PDX cells obtained from a TCRβ-PIM1$^+$ T-LBL case[6] (PDX#3) resulted in pSTAT5 activation and simultaneous upregulation of *PIM1* (**Figure 2B**).

Therefore, the IL7R-JAK-STAT-PIM1 activation status in human T-ALL/T-LBL is defined by a combinaton of both cell intrinsic and cell extrinsic effects affecting this signaling pathway.

Glucocorticoids induce *PIM1* in steroid sensitive T-ALL/T-LBL by transcriptional activation of *IL7RA*

Glucocorticoids, such as prednisone and dexamethasone, are core components of T-ALL treatment[19]. Their mechanism of action is based on binding to the glucocorticoid receptor (NR3C1) and subsequent translocation to the nucleus, where NR3C1 binds to a broad spectrum of specific target genes. Interestingly, previous research showed that glucocorticoids are able to bind an enhancer of the *IL7RA* locus, thereby upregulating *IL7RA* expression in thymocytes[13,14,20].

Given this, we evaluated *IL7RA* expression in our panel of 12 T-ALL/T-LBL PDX samples upon *ex vivo* dexamethasone treatment (100 nM, 24h) in the presence of IL7.

Notably, this analysis confirmed that glucocorticoids can induce *IL7RA* expression in most T-ALL/T-LBL PDX samples (**Figure 3A**). As expected, the one T-ALL patient sample that failed to show *IL7RA* upregulation (PDX#5) was intrinsically resistant to glucocorticoids (**Supplementary Figure 3**).

Finally, we determined whether this glucocorticoid induced *IL7RA* activation would also result in JAK-STAT pathway activation with concamittant upregulation of PIM1. Indeed, *ex vivo* dexamethasone treatment of PDX samples in combination with IL7 caused significant upregulation of both *CISH* and *PIM1* expression along the JAK-STAT axis (**Figure 3B-C**).

Increased levels of PIM1 in T-ALL/T-LBL xenografts upon in vivo induction therapy

To see whether this mechanism of glucocorticoid induced *PIM1* activation would also take place when leukemic cells are exposed to induction therapy in an *in vivo* IL7 producing microenvironment, we performed additional T-ALL/T-LBL xenografts experiments in which we treated mice for one week with a chemotherapeutic cocktail of vincristine, dexamethasone and L-asparaginase (VXL) or vehicle control (**Figure 4A**). After one week, both control and chemotherapy treated mice were sacrificed and $hCD45^+$ leukemic blasts were isolated by FACS from the bone marrow. On average, 2.5 million bone marrow cells were sorted out from control mice, whereas only 100.000-300.000 leukemic cells remained present in the bone marrow after the 4-day chemotherapy regimen. Notably, quantitative RT-PCR revealed significant upregulation of both *PIM1* and *CISH* expression in residual leukemic blasts as compared to controls in 3 $CD127^+$ PDX samples analyzed, confirming that induction chemotherapy can trigger *in vivo* JAK-STAT pathway activation in human T-ALL and T-LBL (**Figure 4B**). The notion that these residual $PIM1^{high}$ leukemia T-ALL/T-LBL cells survived initial debulking by induction chemotherapy might, at least in part, be mediated by glucocorticoid resistance, as recent studies have indeed shown that both cell-intrinsic[21] as well as cell-extrinsic[7-9] mechanisms of JAK-STAT pathway activation can render T-ALL cells less sensitive to steroid therapy. Interestingly, for one PDX sample (PDX#6), we waited for tumor cells to reappear after initial chemotherapy treatment, resulting in re-establishment of disease eight weeks after induction therapy. Notably, *CISH* and *PIM1* levels in these post chemotherapy relapse samples were comparable to the initial levels seen in the tumor xenograft material prior to treatment (**Figure 4C**). Thus, acute chemotherapy triggers *PIM1* induction in residual human T-

ALL/T-LBL cell *in vivo*, but these effects are lost upon disease recurrence after the treatment has been abrogated.

Preclinical evaluation of PIM447 in combination with chemotherapy in a CD127$^+$ T-ALL PDX model that lacks cell intrinsic JAK-STAT pathway activation

Previously, we have shown that *in vivo* combination therapy of a PIM inhibitor with glucocorcticoids resulted in significantly prolonged survival using a PDX model of a TCRβ-PIM1$^+$ T-LBL case[6]. Of note, PDX cells from this T-LBL patient sample have also been included in this study (PDX#3) and these cells showed both cytokine and therapy induced *PIM1* activation. Therefore, the previously published synergistic effects of this combination therapy in PDX#3 are most probably mediated by a combination of both cell intrinsic and cell extrinsic effects that ultimately resulted in robust *in vivo* PIM1 activation.

To further test this, we evaluated the combination of PIM447 and dexamethasone *ex vivo* in PDX cells from both IL7 responder and non-responder T-ALL/T-LBL samples in the presence of exogeneous IL7. Interestingly, synergism was observed in the IL7 responder group, while no beneficial effect was seen in IL7 non-responders (**Figure 5A-B**).

Finally, we evaluated the combination of PIM447 with VXL induction chemotherapy *in vivo* using a PDX sample (PDX#6) that lacked cell-intrinsic genetic abnormalities targeting PIM1 or the IL7R pathway, but had the ability to activate PIM1 upon IL7 stimulation (**Figure 3C**) and induction chemotherapy (**Figure 4B-C**). The treatment schedule is shown in **Figure 5C.** Notably, this analysis revealed a significant improvement in survival in the PIM447/VXL combination group as compared to VXL monotherapy ($p = 0.0103$, **Figure 5D**).

Discussion

We and others have recently shown that PIM1 might serve as a valuable therapeutic target for the treatment of human T-ALL and T-LBL[2-7]. Indeed, *PIM1* translocations[5,6], activating mutations in *IL7RA*, *JAK1*, *JAK3* or *STAT5B*[5,6] or loss-of-function alterations targeting *PTPN2*, can drive cell intrinsic PIM1 activation, rendering human T-ALL/T-LBL cells susceptible to pan-PIM inhibitors such as AZD1208[3] or TP3654[6]. However, non-cell autonomous mechanisms of PIM1 activation might also contribute to the

potential sensitivity of T-ALL/T-LBL cells towards PIM inhibition. Indeed, in this study, we showed that cell extrinsic PIM1 activation by endogenous IL7 can render CD127^{+} T-ALL/T-LBL cells sensitive to *in vivo* PIM inhibition by PIM447. Of note, a recent phase 1 trial in relapsed or refractory multiple myeloma recently revealed that PIM447 is well tolerated in humans suggesting that this agent might have the potential to be translated towards the clinic in the future[18].

Interestingly, the surface IL7R/CD127^{high} T-ALL/T-LBL cells in this study belonged to different molecular genetic subtypes of human T-ALL/T-LBL[21], including both diagnostic and/or relapse samples from immature, *TLX1*$^{+}$, *TLX3*$^{+}$, *HOXA*$^{+}$, *NKX2.1*$^{+}$ and *SIL-TAL1*$^{+}$ cases. In contrast, IL7R/CD127^{low} tumors were TAL/LMO^{+} or NKX2.5^{+}, suggesting that these samples, which lacked the potential for non cell autonomous cytokine induced PIM1 activation, might be enriched for more mature subtypes of human T-ALL/T-LBL.

Although the ability of tumor cells to activate PIM1 upon IL7 stimulation depended on the presence of surface IL7R/CD127 expression[8,9], the actual level of this oncogenic kinase in each T-ALL/T-LBL patient sample will be defined by a combination of cell instrinsic and cell extrinsic effects. Indeed, although IL7R/CD127 levels on tumor cells might initially originate from baseline expression of this surface marker in normal T cell progenitors, additional cell intrinsic genetic defects could be involved in further enhancing *IL7RA* expression in T-ALL/T-LBL cells. For example, loss-of-function alterations targeting *DNM2* will cause impaired clathrin-mediated endocytosis of IL7R/CD127, ultimately resulting in enhanced receptor density on the surface of T-ALL/T-LBL cells[22]. Alternatively, Campos *et al.* recently described a new class of *IL7RA* mutations in human T-ALL that increased the sensitivity of the mutated receptor towards IL7[23]. Furthermore, a recent study also showed that T-ALLs can execute autocrine production of IL7[24], a phenomenon that could further increase IL7 levels in the tumor micro-environment, subsequently causing enhanced PIM1 levels in T-ALL/T-LBL tumor cells. Also in this study, we showed that exogenous IL7 can further increase PIM1 levels in a IL7R/CD127^{+} T-LBL case that already displayed a cell intrinsic TCRβ-PIM1 translocation. With this in mind, we believe that targeting PIM1 might serve as a better and more comprehensive therapeutic strategy for IL7R/CD127^{+} human T-ALL/T-LBL as compared to JAK inhibitors, such as ruxolitinib or tofacitinib. Indeed, although both strategies will be able to target cytokine induced activation of the IL7R-JAK-STAT-PIM1 signaling axis, only the PIM inhibitors will be functional against tumor specific

alterations targeting molecules downstream of JAK proteins, such as STAT5B or PIM1 itself. Alternatively, both therapeutic strategies could also be combined, a notion that is currently evaluated in a trial of PIM447 in combination with ruxolitinib for patients with myelofibrosis (NCT02370706).

Over the last decades, overall survival rates for pediatric T-ALL and T-LBL patients have gradually improved towards almost 90% with the most recent MRD-based treatment protocols. Indeed, patients with a high MRD load after induction therapy receive an intensified treatment regimen potentially followed by a hematopoietic stem cell transplantation. Although this dose escalation strategy has led to improved overall survival rates, it has also been associated with more severe toxic side effects and an increased rate of treatment related mortalities. Therefore, specific therapeutic interventions that could reduce MRD tumor load during induction therapy might serve as valuable therapeutic strategies to reduce the need for therapy intensification, eventually causing less toxic side-effects and potentially lower the relapse rates in human T-ALL or T-LBL.

Here, we used 4 different IL7R/CD127^{+} T-ALL/T-LBL PDX models to show that residual tumor cells, which remain present after 4 days of short-term *in vivo* vincristine – dexamethasone – L-asparaginase (VXL) induction chemotherapy, show consistent upregulation of PIM1 as compared to bulk non-treated tumor cells. Of note, these effects were transient as recurrent disease that reestablished after abrogation of initial short-term *in vivo* chemotherapy again displayed lower *PIM1* expression similar to the levels observed in bulk non-treated control tumor cells. Therefore, these effects are most probably mediated by a direct transcriptional response driven by the short-term chemotherapy regimen itself. In line with this notion, we found that this phenomenon was, at least in part, mediated by the steroids present in the induction therapy treatment regimen. Indeed, in line with previous data showing that glucocorticoids can directly bind a glucocorticoid response element upstream of the *IL7R* TATA box are in human CD4+ and CD8+ T-cells[16], we showed that dexamethasone treatment in T-ALL/T-LBL PDX cells resulted in transcriptional upregulation of *IL7RA*. Of note, recent studies have shown that both cell-intrinsic[21] as well as cell-extrinsic[7-9] mechanisms of JAK-STAT pathway activation can render T-ALL cells less sensitive to steroid therapy. Therefore, one could hypothesize that this therapy induced *IL7RA* activation eventually leads to non-cell autonomous stimulation of the JAK-STAT-PIM1 signaling pathway by endogenous IL7, ultimately resulting in residual PIM1^{high} T-ALL/T-LBL cells that survive

initial debulking through their reduced sensitivity towards glucocorticoids. With this in mind, we finally showed that *ex vivo* combination therapy of PIM447 and dexamethasone is synergistic in IL7R/CD127$^+$ T-ALL and T-LBL PDX cells. In addition, using a PDX model from a IL7R/CD127$^+$ T-ALL that lacked cell-intrinsic IL7R-JAK-STAT-PIM1 abnormalities, we confirmed in vivo that combination PIM447 with VXL induction chemotherapy improves leukemia survival.

Altogether, our study shows that cytokine and therapy induced PIM1 activation can be therapeutically targeted by the pan-PIM inhibitor PIM447 in human T-ALL/T-LBL. In addition, our work suggests that CD127 expression could serve as a valuable biomarker in human T-ALL/T-LBL to stratify patients that might benefit from PIM inhibition during induction chemotherapy.

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Authorship Contributions

RDS, LR, JM, VB, JTB, RLS, SG and AA performed experiments. RDS, PVV, JR, CM, TT and WVL performed analyses. LR and BL provided technical assistance. LC, RK, MM, AU, PV, TL, NVR and BDM collected and provided primary T-ALL patient material. PVV and RDS designed research and wrote the paper, with help from the other authors. All authors have seen, reviewed and approved the final version.

Conflicts of Interest

No conflicts of interest

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Figure Legends

Figure 1. A subset of T-ALL/T-LBL patients upregulate *PIM1* expression in response to IL7. (A) Patient derived xenograft (PDX) spleen samples were stimulated with 100 ng/ml IL7 for 30 minutes, after which phosphorylation sites were fixed with methanol and phosphorylated STAT5 (Tyr 694) was measured by flow cytometry. Fold changes (FC) of the mean fluorescent intensity of phosphorylated STAT5 (Tyr 694) are shown for 12 PDX spleen samples. **(B)** *IL7R* expression levels, measured by RT-qPCR, for 12 PDX spleen samples. *CNRQ = Calibrated Normalized Relative Quantities* **(C)** PDX spleen samples were stimulated with 50 ng/ml IL7 for 24 hours and were subsequently collected for RNA isolation. Fold changes (IL7/no IL7) of *PIM1* expression levels are shown, measured by RT-qPCR. **(D)** A PDX model of PDX#6 was established, after which 3 mice per group were treated for two weeks (5 days on/2 days off) with either 30 mg/kg AZD1208 or 100 mg/kg PIM447 or their respective vehicles. Percentages of human CD45 in peripheral blood are shown per group for three timepoints. **(E)** PDX#6 mice were sacrificed after 2 weeks of treatment with either AZD1208 or PIM447. Spleen weights are shown per treatment group. **(F)** *Ex vivo* treatment of PDX#6 spleen cells. 50.000 cells were treated per condition for 72h in 10% RPMI supplemented with 10 ng/ml IL7, 50 ng/ml SCF, 20 ng/ml FLT3 and 100 ng/ml IL2, in duplicate. ATP content was measured by means of a CellTiter-Glo viability assay.

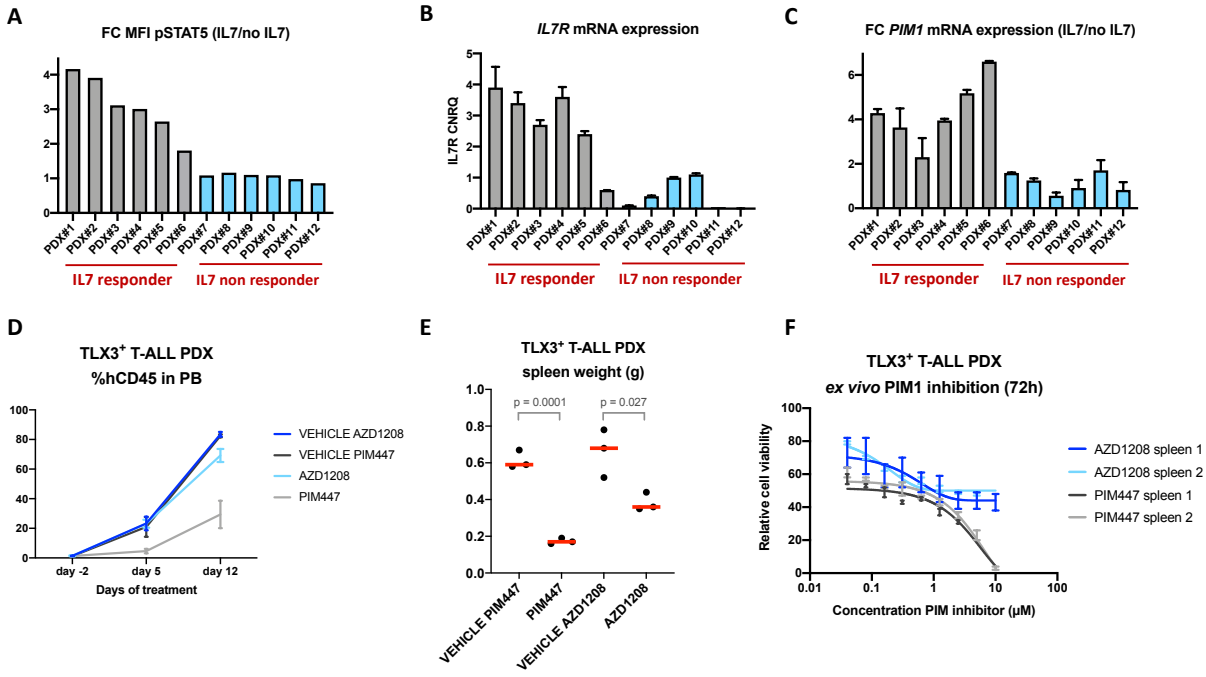


Figure 2. Intrinsic and extrinsic *PIM1* activation can co-occur. (A) HPB-ALL cells were stimulated with 100 ng/ml IL7 for 30 minutes and stained for phospho-STAT5 (Tyr 694) conjugated to APC (left). For downstream differential transcriptional analysis via *PIM1* RT-qPCR, cells were stimulated with 50 ng/ml IL7 for 24 hours (right). **(B)** PDX#3 spleen cells were stimulated with 100 ng/ml IL7 for 30 minutes and stained for phospho-STAT5 (Tyr 694) conjugated to APC (left). For downstream differential transcriptional analysis via *PIM1* RT-qPCR, cells were stimulated with 50 ng/ml IL7 for 24 hours (right).

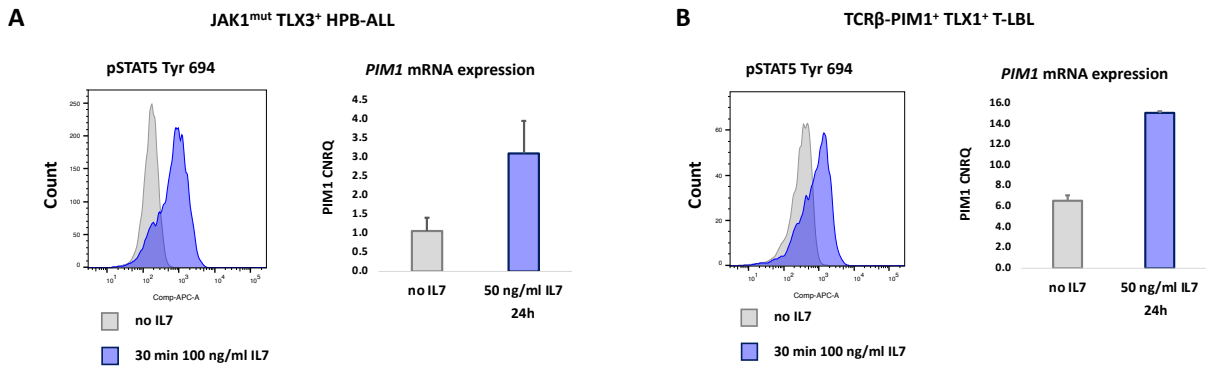


Figure 3. Glucocorticoid responsive T-ALL/T-LBL PDX samples show *IL7R*/*CISH*/*PIM1* upregulation upon dexamethasone treatment. (A) *IL7R* mRNA expression levels are shown for 12 PDX spleen samples before and after 100 nM dexamethasone treatment for 24h, in 10% RPMI supplemented with 50 ng/ml IL7. **(B)** *CISH* mRNA expression levels are shown for 12 PDX spleen samples before and after 100 nM dexamethasone treatment for 24h, in 10% RPMI supplemented with 50 ng/ml IL7. **(C)** *PIM1* mRNA expression levels are shown for 12 PDX spleen samples before and after 100 nM dexamethasone treatment for 24h, in 10% RPMI supplemented with 50 ng/ml IL7.

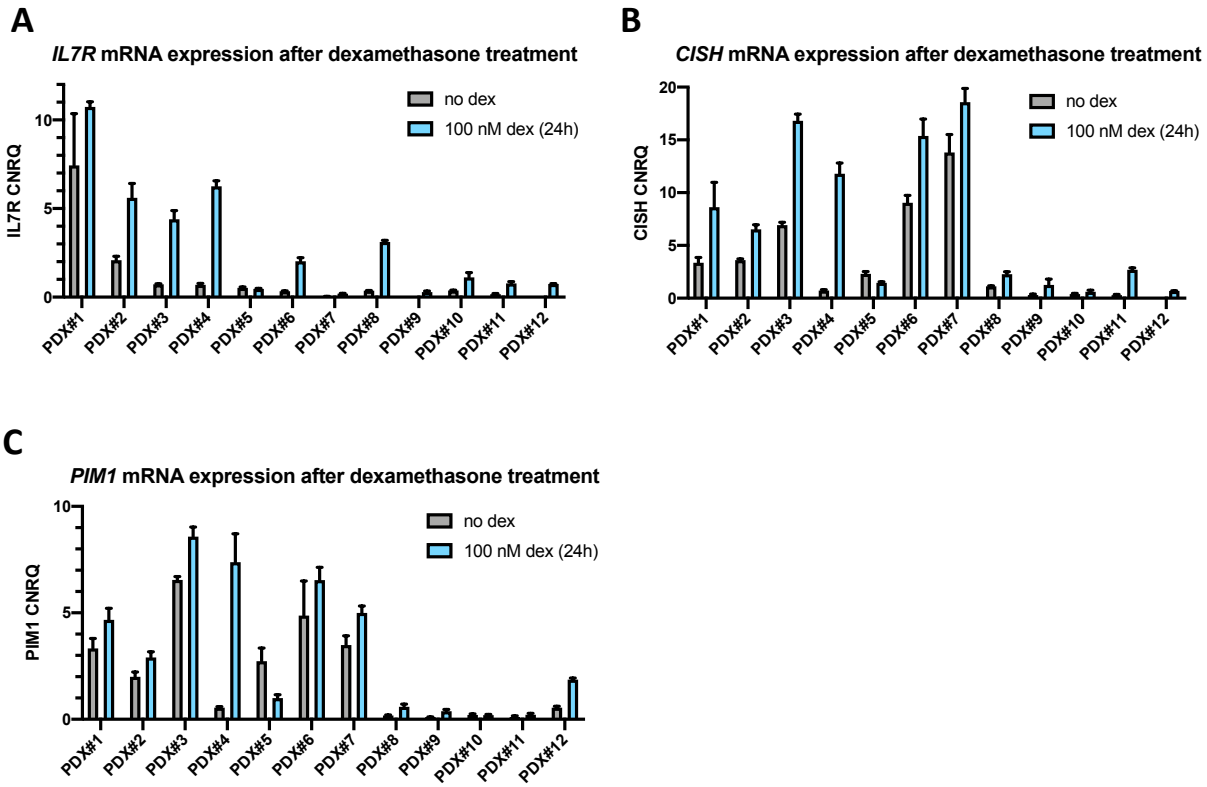


Figure 4. Chemotherapy resistant blasts are characterized by high *PIM1* expression. (A) *In vivo* chemotherapeutic treatment schedule. 4 mice per group per PDX were treated with a chemotherapeutic cocktail via i.p. and sacrificed upon day 5. Leukemic blasts (hCD45^{+} cells) were sorted out from bone marrow, after which RNA was isolated. **(B)** RT-qPCR for *CISH* and *PIM1* on remaining blasts in the bone marrow after *in vivo* chemotherapy treatment. **(C)** RT-qPCR for *CISH* and *PIM1* on bone marrow samples from non treated (vehicle) mice, mice that were treated for 1 week with the chemotherapeutic cocktail from **Figure 4A** (chemo) and mice that relapsed again after that relapsed after one week of chemotherapy treatment (chemo relapse). *CNRQ = Calibrated Normalized Relative Quantities*

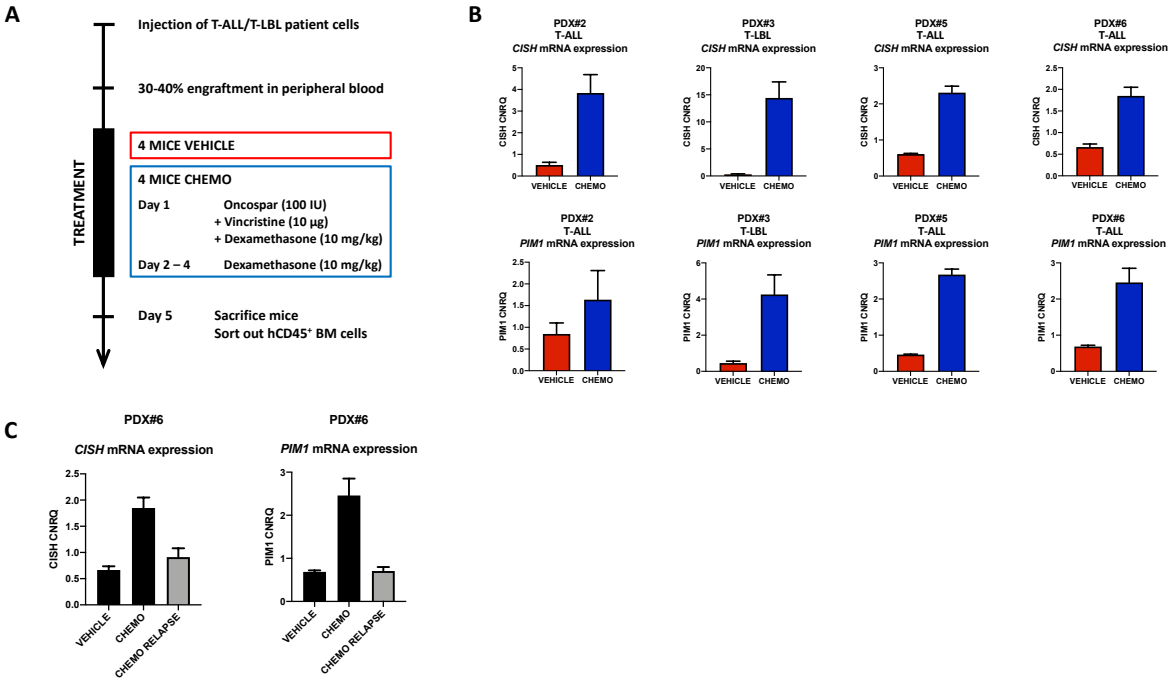
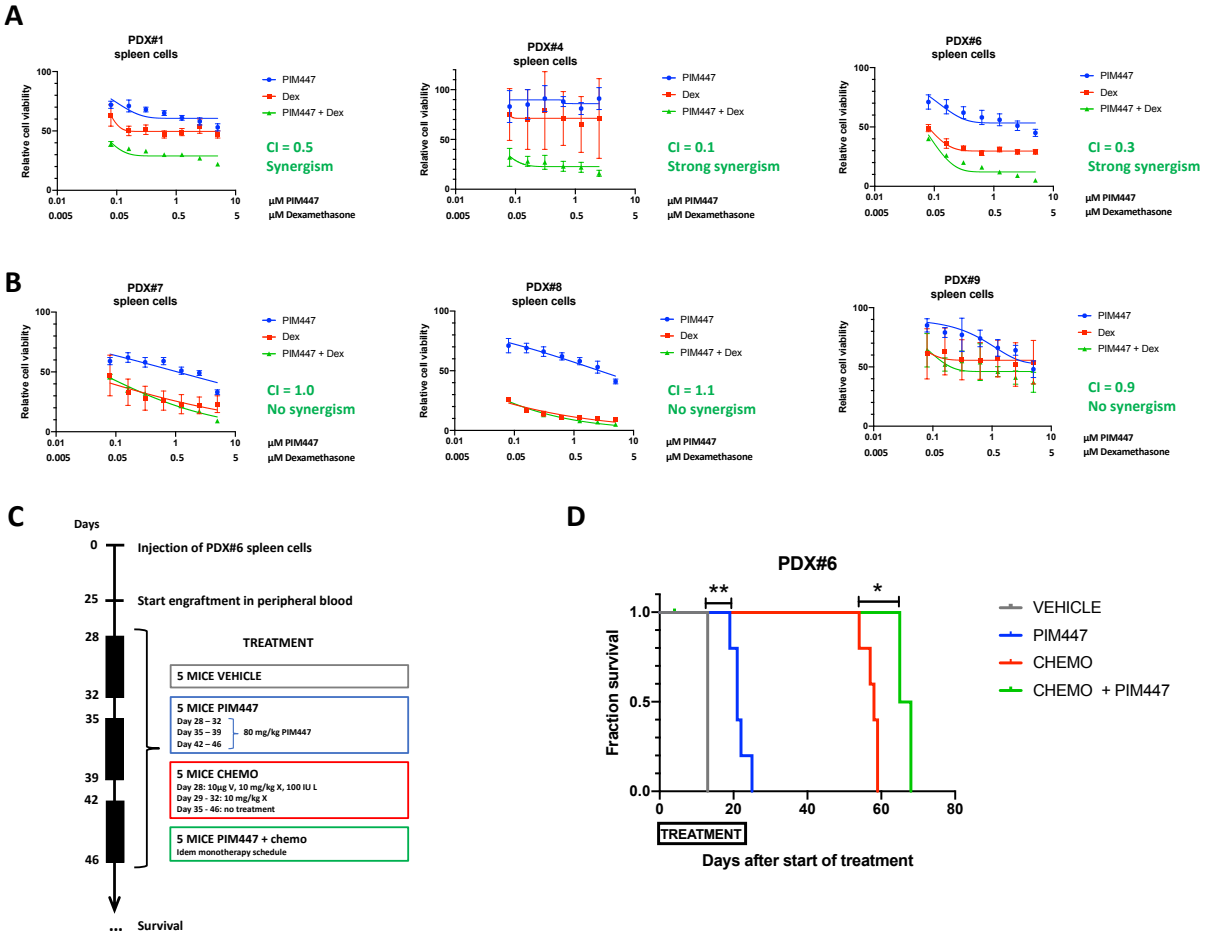
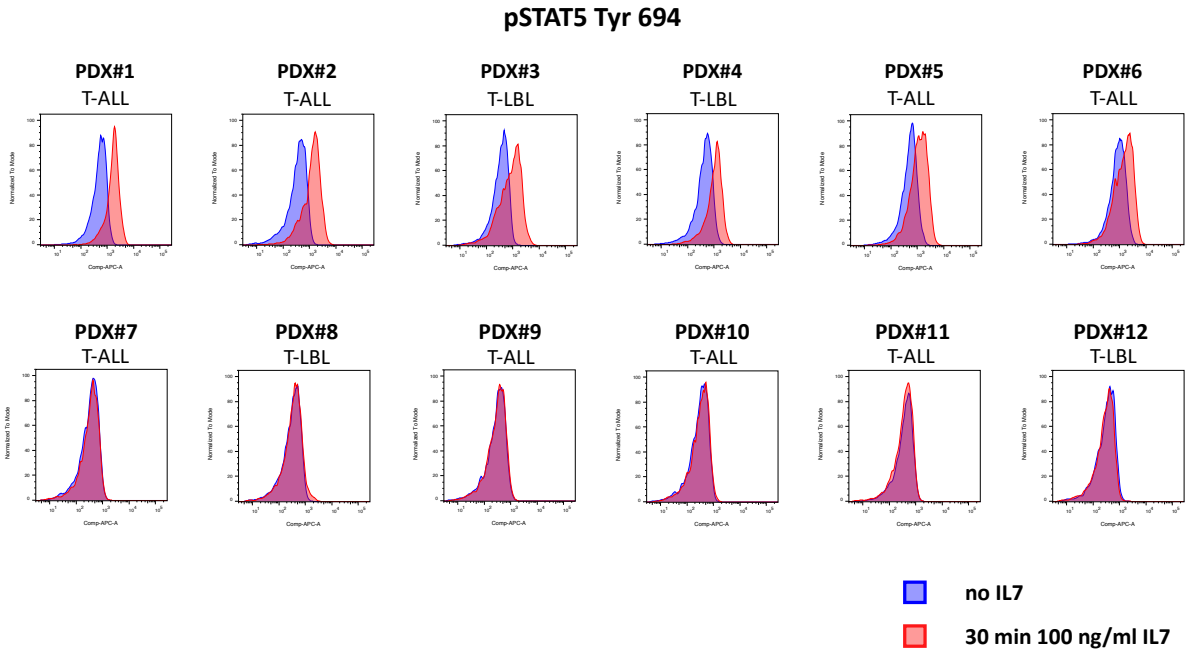


Figure 5. PIM447 – glucocorticoid combination therapy works synergistic in IL7 responsive T-ALL/T-LBL. (A) *Ex vivo* combination treatment with PIM447 and dexamethasone of IL7 responsive PDX spleen cells. 50.000 cells were treated per condition for 72h in 10% RPMI supplemented with 10 ng/ml IL7, 50 ng/ml SCF, 20 ng/ml FLT3 and 100 ng/ml IL2, in duplicate. ATP content was measured by means of a CellTiter-Glo viability assay. Combination indexes (CI) were calculated with CalcuSyn software. **(B)** *Ex vivo* combination treatment with PIM447 and dexamethasone of IL7 non responsive PDX spleen cells. The same conditions were used as explained in **5A**. **(C)** *In vivo* combination treatment schedule with PIM447 and chemotherapeutics. The first week, 5 mice per group were treated with either vehicle, or 80mg/kg PIM447 through oral gavage, or the chemotherapeutic cocktail through intraperitoneal injection, or the combination of PIM447 with chemo. The second and third week, only 80mg/kg PIM447 was administered. The frequency of treatment was once daily for 5 days on, 2 days off. Mice were monitored for survival and were sacrificed when weight dropped more than 20%, leukemic blasts in the blood reached 90% or when mice looked too ill (bad fur, heavy breathing, slowly responsive). V = vincristine, X = dexamethasone, L = L-asparaginase **(D)** Survival curve for *in vivo* treatment of PDX#6 with PIM447 and chemotherapeutics. * = p < 0.05, ** = p < 0.01



Supplemental data

Supplemental Figure 1. Phospho-STAT5 (Tyr694) levels before and after 30 minutes 100 ng/ml human IL7 stimulation in 10% RPMI for the 12 PDX spleen samples.



Supplemental Figure 2. Cell cycle and apoptosis assays of PDX3 spleen cells before and after treatment with 1µM PIM447 for 48h in 10% RPMI supplemented with 50 ng/ml IL7. Annexin V positive cells depict early apoptotic cells, whereas propidium iodide – Annexin V double positive cells depict late apoptotic cells.