Triggering the TCR developmental checkpoint activates a therapeutically targetable tumor suppressive pathway in T-cell leukemia

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A.T., N.d.S. and C.T.Q. designed and performed experiments, analyzed the data and helped write the manuscript. FR and BZ participated in the experiments of Figure 7 and Suppl Fig 3. M.B. and S.S. performed transcriptomic analysis. C.D.J. performed xeno-transplantation experiments and analyzed data. L.L., M.T. contributed to phosphokinase array analysis. D.G. provided mice spleens, the TCR-HY vector, peptides and expertise for in vitro TCR stimulation by antigen presenting cells. F.L.C and E.L provided essentials reagents. L.C. provided murine CD3 monoclonal antibody and expertise in anti-CD3 treatment. M.D. provided technical expertise in imaging cytometry. F.P., H.D. and N.I. provided human T-ALL samples. D.G., E.M. and O.H.
contributed to the writing and editing of the manuscript. V.A. and J.G. designed, supervised the project, analyzed the data and wrote the manuscript.

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ABSTRACT

Cancer onset and progression involves the accumulation of multiple oncogenic hits, which are thought to dominate or bypass the physiological regulatory mechanisms in tissue development and homeostasis. We demonstrate in T-cell acute lymphoblastic leukemia (T-ALL) that, irrespective of the complex oncogenic abnormalities underlying tumor progression, experimentally induced, persistent TCR signaling has anti-leukemic properties and enforces a molecular program resembling thymic negative selection, a major developmental event in normal T cell development. Using mouse models of T-ALL, we show that induction of TCR signaling by high affinity self-peptide/MHC or treatment with monoclonal antibodies to the CD3ε chain (anti-CD3) causes massive leukemic cell death. Importantly, anti-CD3 treatment hampered leukemogenesis in mice transplanted with either mouse or patient-derived T-ALLs. These data provide a strong rationale for targeted therapy based on anti-CD3 treatment of TCR-expressing T-ALL patients and demonstrate that endogenous developmental checkpoint pathways are amenable to therapeutic intervention in cancer cells.

SIGNIFICANCE: T-ALL are aggressive lymphoid malignant proliferations of T-cell precursors characterized by high relapse rates and poor prognosis, calling for the search of novel therapeutic options. Here we report that the lineage-specific TCR/CD3 developmental checkpoint controlling cell death in normal T cell progenitors remains switchable to induce massive tumor cell apoptosis in T-ALL and is amenable to pre-clinical therapeutic intervention.
INTRODUCTION

Developmental checkpoints in stem/progenitor cells are critical to their determination, commitment and differentiation into distinct lineages. Cancer cells often retain expression of lineage-specific checkpoint proteins, but their potential impact in cancer remains elusive (1). T lymphocytes mature in the thymus following a highly orchestrated developmental process that entails the successive rearrangements and expression of T-cell receptor (TCR) genes. TCRδ rearrangements first occur at the CD5+, CD1a–, CD4/8 double negative (DN) stage, followed by concurrent TCRγ and TCRβ rearrangements coinciding with CD1a expression. While productive TCRγ and TCRδ rearrangements will determine the assembly of a TCRγδ, a complete productive TCRβ gene rearrangement will first allow surface expression of a pre-TCR complex formed by the assembly of the TCRβ chain with a pre-Tα (pTα) invariant chain. Pre-TCR surface expression is a critical checkpoint for developing αβ T-cell precursors to expand and mature into CD4/CD8 double positive (DP) cortical thymocytes and for the initiation of Vα-Jα rearrangements. These rearrangements continue until a TCRα chain is formed that can associate with the already formed TCRβ chain to assemble a complete mature TCRαβ at the cell surface (2, 3). Cell surface expression of the TCR is a critical developmental checkpoint in establishing MHC restriction and in shaping the immunological repertoire. Low affinity recognition of self-peptide/Major Histocompatibility complexes (self-pMHC) presented by thymic epithelial cells to the TCR of CD4+CD8+ double positive (DP) cortical thymocytes transduces positive selection signals and commitment to either the CD4 or CD8 lineages. DP thymocytes not receiving these signals die by lack of stimulation whereas those that recognize self-pMHC with high affinity undergo TCR-mediated apoptosis and negative selection (4, 5).
T-cell acute lymphoblastic leukemia (T-ALL), results from the leukemic transformation of thymic cell precursors and their arrest at specific stages of differentiation (6-8). Despite recent and extensive insights into the molecular and cellular mechanisms responsible for T-ALL onset and progression, survival rates remain around 50 and 70% in adult and pediatric cases, respectively, calling for the search of novel therapeutic options. In T-ALL, leukemic transformation of maturating thymocytes is caused by a multistep pathogenesis involving numerous genetic abnormalities that drive normal T cells into uncontrolled cell growth and clonal expansion. T-ALL is classified into subgroups based upon exclusive gene rearrangement and/or expression of limited set of transcription factors including TAL1, LMO1/2, TLX1/3, associated with the specific arrest at distinct stages of T cell differentiation (8-10). Across these molecular subclasses, a number of other recurrent genetic alterations are found, including inactivation of the CDKN2A tumor suppressor locus, NOTCH1 pathway activating mutations and many others (reviewed in (11)).

In this study, we hypothesized that tissue homeostatic regulators may be amenable to reactivation in tumor cells and demonstrate that experimentally induced TCR signaling in T-ALL induces cell death and a molecular program similar to negative selection of thymic T-cell progenitors. Importantly, switching-on this TCR-induced cell death program dominates the multiple anti-apoptotic and pro-proliferative mechanisms controlled by the oncogenes and altered tumor suppressors of T-ALL. These data provide strong rationale for targeted therapy based upon anti-CD3 treatment in TCR-expressing T-ALL.
RESULTS

T-ALL development is hampered by antigen presenting cell-mediated TCR stimulation

To investigate the role of TCRαβ activation by a defined antigen in T-ALL, we stably expressed the negatively-selecting Marilyn TCR-HY (Vα1.1Vβ6) specific for the HY male antigen (DBY) in the TCR-negative ALL-SIL T-ALL cell line. In vitro co-culture of ALL-SIL-TCR-HY cells with splenocytes pulsed with increasing doses of DBY peptide, but not the unrelated OVA peptide, induced leukemic cell death (Fig. 1A and quantified in Fig. 1B). DBY peptide did not induce apoptosis of parental TCR-negative ALL-SIL cells nor of ALL-SIL-TCR-HY cells cultured in absence of MHC-restricted antigen-presenting syngeneic splenocytes (Fig. 1A). These data demonstrate that TCR activation by the cognate peptide-MHC complex results in T-ALL cell death. To determine the effect of persistent TCR stimulation on T-cell leukemogenesis, we generated double transgenic mice (hereafter referred as TEL-JAK 2/TCR-HY mice) expressing the TEL-JAK2 fusion oncogene (12) and the Marilyn TCR-HY (13) in lymphoid progenitors. As compared to TEL-JAK2/TCR-HY double transgenic females, which developed rapid and fully penetrant T-ALL, the majority of TEL-JAK2/TCR-HY males either failed to become leukemic or developed leukemia with markedly delayed latency (Fig. 2A). Flow cytometry analysis showed that of seven diseased male mice analyzed, two developed B-cell lymphoma/leukemia while five developed T-cell leukemia (Fig. 2B). Strikingly, in the five T-ALL cases, transgenic TCR-Vβ6 cell surface expression was low or absent and leukemic cells often expressed either no TCR or an endogenous TCR (Fig. 2C), suggesting that in these mice, expression of TCR-HY, but not non-HY-specific TCR complexes was counterselected during leukemogenesis. We conclude that self-
antigen TCR stimulation suppresses thymocyte malignant transformation, thus delaying leukemogenesis in male mice.

To investigate whether delayed leukemogenesis in TEL-JAK2/TCR-HY male mice could be attributed to direct antigenic stimulation of TCR-expressing leukemic cells, female TEL-JAK2/TCR-HY CD45.1+ T-ALL cells were transplanted to either female (HY-negative) or male (HY-positive) syngeneic CD45.2+ recipient mice. As compared to female recipients, which rapidly developed fatal T-ALL, most recipient males failed to develop leukemia and survived longer (Fig. 2D). In contrast to male mice sacrificed simultaneously, female recipients presented high infiltration of CD45.1+ leukemic cells in spleen, lymph nodes, and bone marrow (Fig. 2E and F, and data not shown). The resistance of male mice to TEL-JAK2/TCR-HY-induced leukemogenesis depended on HY antigen MHC presentation but not upon an adaptive immune response, since T-ALL developed efficiently following transplantation of TEL-JAK2/TCR-HY leukemic cells in male allogeneic immunodeficient recipient mice (Fig. 2G), but not in male syngeneic immunodeficient recipients (Fig. 2H). These results show that the TCR anti-leukemic effect depends upon its stimulation by the cognate peptide-MHC complex.

**Anti-CD3 stimulation of ALL-SIL/TCR-HY cells induces TCR signaling and apoptosis.**

We next investigated whether anti-CD3 treatment could mimic TCR signaling to induce leukemic cell apoptosis. Unlike control ALL-SIL cells, TCR-HY-expressing cells underwent massive apoptosis when treated with anti-CD3/anti-CD28 or anti-CD3 alone (data not shown), as revealed by increased levels of cell surface AnnexinV, increased cleavage of caspase 3 and 7 and reduced cell expansion (Fig. 3A–D). Importantly, anti-CD3/anti-CD28 treatment did not impact on cell cycle progression _per se_ (Fig. 3E and data not shown). To investigate the signaling properties of the mouse TCR-HY complex in human leukemic ALL-SIL cells, we first demonstrated that CD3
crosslinking induced TCR clustering at the ALL-SIL cell surface (Fig. 3F). Next, phosphokinase-array analysis of anti-CD3/anti-CD28-stimulated ALL-SIL/TCR-HY and normal human DP thymocytes showed increased phosphorylation of overlapping TCR signaling components, including the key downstream second messengers ERK, JNK and AKT (Fig. 3G). Anti-CD3-induced phosphorylation of these proteins, and not of the cytokine signaling STAT3 protein, was confirmed by flow cytometry (Fig. 3H and data not shown). Of note, inhibition of TCR signaling by cyclosporine or the MEK kinase inhibitor PD184352 impaired anti-CD3-induced apoptosis and expression of the CD69 and CD25 T-cell activation markers (Fig. 3I and J). Together these results indicate that the TCR/CD3 signaling module is functional in T-ALL cells, and that its activation leads to apoptosis. Importantly, similar results were observed in primary T-ALL (Supplementary Figure 1A-C).

Targeting CD3 in vitro induces human primary T-ALL apoptosis and mimics thymic negative selection

To test whether anti-CD3 stimulation of TCR-expressing T-ALL diagnostic samples also induced apoptosis, primary human T-ALL cells (see Suppl. Table1 for the immunophenotypic and genetic features of the diagnostic samples used) belonging to the major molecular oncogenic T-ALL subclasses were treated with anti-CD3/anti-CD28. Massive T-ALL apoptosis and TCR signaling activation were observed in TCR-expressing T-ALL but not in TCR-negative cases, regardless of their molecular oncogenic subtype (Fig. 4A–D). We next analyzed the transcriptional profiles of TCR-expressing ALL-SIL cells either unstimulated or after TCR stimulation and found in the ALL-SIL TCR-induced transcriptional profile a significant enrichment for the TCR-induced negative selection signature of normal thymic progenitors (14) (Fig. 4E and F). This shows that
T-ALL apoptosis induced by TCR stimulation is associated with signaling cues resembling those of TCR-induced negative selection in normal T cell progenitors.

**Mouse and human T-ALL development is hampered by TCR stimulation with agonistic monoclonal antibodies**

To assess the therapeutic potential of TCR stimulation by anti-CD3 treatment *in vivo*, we first inoculated female syngeneic mice with transgenic TEL-JAK2/TCR-HY leukemic cells and the following day initiated treatment with either an anti-CD3ε monoclonal antibody (mAb) known to induce negative selection in normal thymic progenitors (15) or an isotypic control antibody for 5 consecutive days. Strikingly, both peripheral blood leukemia and fatal disease were impaired in anti-CD3-treated mice while all mice treated with control antibody rapidly succumbed to leukemia (Fig. 5A and B). Importantly, treatment with anti-CD3 mAb was also effective against TEL-JAK2 T-ALL cells expressing endogenous surface TCRαβ (Fig. 5C and D). Of note, anti-CD3 treatment only marginally prolonged the leukemia-free survival of mice infused with CD3ε−/− TEL-JAK2 T-ALL cells (Fig. 5D). This shows that the extended survival seen in anti-CD3-treated mice inoculated with CD3ε+/+ TEL-JAK2 T-ALL (Fig. 5D) critically depends upon engagement of the TCR/CD3 complex expressed by leukemic cells. To ensure that the results were not restricted to this particular model of activated tyrosine-kinase-induced T-ALL, we extended these analyses to *Ikzf1/Ikaros*-mutated-induced T-ALL. Although *Ikaros* is rarely found mutated in T-ALL, recent evidence points to a suppressive function of Ikaros in NOTCH-mutated T-ALL cells (16). Treatment with anti-CD3 mAb inhibited leukemia expansion in this model (Fig. 5E), which translated into increased survival of anti-CD3-treated mice (Fig. 5F).
Finally and most importantly, we investigated whether anti-CD3 treatment also displayed anti-leukemic activity against human CD3-positive T-ALL diagnostic cases. Mice were xenotransplanted with cells from seven distinct human T-ALL cases, five of them expressing a TCR at the cell surface, one (UPNT374) showing an heterogeneous TCR expression pattern and the last one (UPNT525) being negative for TCR cell surface expression. Mice were treated 24 h later with either anti-CD3 mAb or an isotypic control antibody. For all TCR⁺ T-ALL samples, anti-CD3 treatment showed strong anti-leukemic effects, as shown by delayed dissemination in blood, bone marrow and other organs (Fig. 6A, B, C and D), an effect that translated into enhanced recipient mouse survival (Fig. 6E and Suppl. Fig. 2). In contrast, leukemic mice inoculated with TCR-negative T-ALL cells were insensitive to anti-CD3 treatment (Fig. 6B and Suppl. Fig 2). Mice infused with UPNT374 cells only transiently responded to anti-CD3 (Fig. 6B and Suppl. Fig. 2). Interestingly, leukemic cells recovered from anti-CD3-treated mice showed low or absence of TCR cell surface expression in contrast to the leukemic cells that expanded in control IgG-treated mice (Fig 6F). This confirms the specific depletion of TCR-positive leukemic cells in response to TCR activation. We next investigated whether anti-CD3 treatment could also result in anti-leukemic effects in a curative setting. First, NSG mice were inoculated with the luciferase-expressing human ALL-SIL/TCR-HY cells and then treated with either a control or an anti-CD3 mAb when mice presented leukemia engraftment in BM (day 22 post-inoculation, data not shown). Comparison of the two groups by live imaging showed a specific anti-leukemic effect of anti-CD3 treatment (Fig. 7A, top panels and 7B). To investigate whether anti-CD3 anti-leukemic effects were dependent upon TCR signaling, we sought to silence the expression of LAT, a critical adaptor in TCR signaling in thymocytes and mature T cells (17). Small hairpin (sh) RNAs to LAT resulted in LAT expression knock-down in ALL-SIL/TCR-HY cells (Suppl. Fig. 3A) and had no effect on TCR cell surface expression (Suppl. Fig. 3B). LAT knock-down
impaired anti-CD3-induced *in vitro* apoptosis (Suppl. Fig. 3C) and anti-leukemic effects *in vivo* (Fig.7A, B and Suppl. Fig.3 D, E). As expected, LAT expression knockdown was maintained in leukemic cells retrieved from control and anti-CD3 mAb treated mice (Suppl. Fig. 3F). In a second experiment, mice xenotransplanted with cells from T-ALL UPNT419 were treated with the anti-CD3 or control mAb when leukemic cell burden reached 10-20% of bone marrow cells. Leukemia expansion was strongly delayed in anti-CD3-treated mice in contrast to the rapid expansion of leukemic cells in control mice (Fig. 7C). Despite the re-emergence of peripheral blood leukemic cells upon cessation of treatment, survival of anti-CD3-treated mice was significantly prolonged (Fig. 7D). Thus, antibody-mediated TCR engagement results in striking anti-leukemic effects in xenotransplanted human T-ALLs in both preventive and curative settings.
DISCUSSION

Current T-ALL therapies involve complex, often toxic chemotherapeutic regimens. Although T-ALL outcome has improved with current therapies, survival rates remain only around 50 and 70% at 5 years in adult and pediatric T-ALL, respectively (18, 19). The genetic bases of T-ALL progression and maintenance are well characterized but have not translated so far into targeted therapies (11). There is thus unmet need for new treatments to offer therapeutic options for refractory disease and to prevent relapse. We previously observed that expression of a TCR transgene in a TCR-negative T-ALL cell line can induce cell death, provided these cells are subjected to differentiation inducing cues (20). We report here that chronic/strong TCR signaling causes massive apoptosis of primary T-ALL that express an endogenous TCR and shows potent tumor suppressive function in vivo. These findings call for the incorporation of TCR-directed therapies in current treatment regimens of CD3-expressing T-ALL. Muromonab-CD3 (OKT3) was approved by the U.S. Food and Drug Administration (FDA) in 1985 for therapy of acute, glucocorticoid-resistant rejection of allogeneic renal, heart and liver transplants (21) and was in fact the first monoclonal antibody introduced in the clinic. Since then, a number of other monoclonal antibodies to CD3 were developed (22) that may prove superior to OKT3 in T-ALL treatment. Of note, encouraging response to OKT3 therapy was reported in an adult patient with an aggressive and chemotherapy-resistant T-ALL, but the basis of this response was not studied (23).

A major drawback of current chemotherapeutic regimens in T-ALL is the frequent resistance to treatment and relapse. Leukemia initiating cells (LICs) from residual disease are thought to be responsible for relapsing cases (24, 25). In addition, resistance of T-ALL to
chemotherapy is in part linked to the recurrent genetic abnormalities selected during disease progression, e.g. inactivation of the PTEN tumor suppressor gene and the resulting activation of PI3kinase/AKT signaling (26, 27). Whether administration of anti-CD3 therapy during the remission phase or its association with conventional chemotherapy regimens could target LICs and/or bypass molecular mechanisms of primitive resistance represent promising directions to be explored in future prospective studies.

T cells mature in the thymus following a highly orchestrated process controlled both by cell intrinsic (e.g. transcription factors) and extrinsic (e.g. stroma-derived cytokines/chemokines) molecular cues (28, 29). Cell surface TCRαβ expression in DP thymocytes allows recognition of specific self-MHC/peptide to transduce a positive selection signal and maturation into SP thymocytes. DP thymocytes not receiving this signal die through lack of stimulation; whereas those whose TCR binds too strongly to self-MHC/peptide undergo activation-induced apoptosis and negative selection (5, 30). In both situations TCR binding to pMHC is the triggering event but how TCR engagement leads to such divergent outcomes (survival and proliferation versus death) remains unclear (31). Remarkably, these two contrasting processes are driven by a TCR signaling machinery of qualitatively similar composition (30). Available evidence indicates that the difference lies in the molecular interpretation of signals of different strength, which may rely on compartmentalization of key signaling players (32) and in the induction of divergent, complex transcriptional response (14, 33). Indeed, it has been shown that a small increase in ligand affinity for the TCR leads to a marked change in the subcellular localization (plasma membrane for negative selecting ligands versus Golgi complex for positively selecting ones) of essential adaptors of the Ras signaling pathway. This compartmentalization induces the conversion of a small change in analogue input (affinity for ligand) into a digital output (positive versus negative
selection) (32, 34). Our results show that the anti-leukemic activity of anti-CD3 mAb depends upon TCR signaling cues as it was impaired upon LAT silencing in T-ALL cells. This also suggests that, in the present experimental setting, ADCC (Antibody Dependent Cell Cytotoxicity)-like responses of the host play a minor role in anti-CD3 anti-leukemic properties. It is likely that in T-ALL patients eligible for anti-CD3 therapy (about half of pediatric and 30% of adult cases), ADCC-like responses will play a more important role than in immunosuppressed mice and will add therapeutic value to the intrinsic anti-leukemic effects of anti-CD3. In addition, in a clinical context, TCR down-regulation and/or selection of a TCR-negative subclone, as observed here in a xenotransplanted T-ALL under pressure of anti-CD3 treatment could constitute a potential escape mechanism to treatment. These findings need to be explored in clinical trials. More extensive studies on the composition and function of TCR-induced signalosomes formed in T-ALL stimulated by anti-CD3 and the identification of critical components in T-ALL of the transcriptomic signature akin to that of thymic negative selection (14, 35, 36) should provide information on the molecular pathways involved in anti-CD3 induced apoptosis in leukemic cells. These pathways could in turn constitute new pharmaceutical targets to treat T-ALL.

Although signaling from the B-cell receptor (BCR) and pre-BCR was shown to promote B-cell malignancies (37, 38), the role of TCR signaling in T-ALL has thus far remained controversial. Studies in T-ALL mouse models indicated a pro-oncogenic role for TCR signaling (39-42). However, these data were based on transgenic TCR systems, which do not reflect the expression levels and receptor diversity found among T-ALL patients where TCR expression is heterogeneous or absent (6). Moreover, gene inactivation studies in other mouse T-cell leukemia models have shown that TCR expression is not essential for T-ALL development (43, 44). The current work demonstrates that anti-CD3-mediated activation of endogenous TCR in human T-
ALL has an anti-leukemic function. T-ALL often arises from immature T-cell precursors before the stage of negative selection (6, 42). Consequently, contingent expression of a non-negatively selected TCR will render T-ALL cells sensitive to TCR-activating apoptotic signals that mimic negative selection, as demonstrated here by anti-CD3 treatment \textit{in vitro} and \textit{in vivo}. However, during T-ALL development, it is possible that selective events enable cells to escape post-malignancy negative selection, such as loss of TCR surface expression, as found in a subset of T-ALL patients (6) and as demonstrated here in TEL-JAK2/TCR-HY double transgenic male mice. Such leukemias could be amenable to other therapies targeting downstream TCR signaling effectors.

The dual role of developmental molecular pathways in organogenesis and tumorigenesis is increasingly recognized, the modulation of which may provide potential therapeutic opportunities (1). In line with this, experimental restoration of cell surface expression of Igα and Igβ in Ph⁺ B-ALL was also shown to result in cell death (45). In our study, we found that reactivation in TCR-positive T-ALL blasts of the lineage-specific checkpoint control normally set by TCR signaling during T-cell development, displays anti-tumoral functions. Importantly, despite the multiple and complex oncogenic mechanisms driving T-ALL, which include anti-apoptotic (46) and pro-proliferative (47) signaling cues, this TCR-dependent checkpoint remains switchable to induce massive tumor cell apoptosis. Thus, reactivation of similar lineage-specific developmental checkpoints in malignancies originating from other lineages and tissues could provide a novel class of therapeutic targets in cancer.
Datasets generated for this study have been deposited in Gene Expression Omnibus (GEO) database with accession number GSE65496.

MATERIALS AND METHODS

Mice. TEL-JAK2 mice were bred with Marilyn transgenic, Rag2-deficient or CD3ε-deficient mice (all maintained on the C57BL/6 background), as described previously (43). Swiss Nude and NOD/SCID/γc−/− (NSG) mice were purchased from Charles River Laboratories. IKL/L mice, (carrying two alleles of the hypomorphic IKL allele (48) were a generous gift of Dr. P. Kastner, (IGBMC, Strasbourg, France). Mice were maintained under specific-pathogen-free conditions in the animal facilities of the Institut Curie (Orsay, France) or University of Algarve (Faro, Portugal). NSG mice were maintained under constant antibiotic treatment (Baytril 0.01% in drinking water). 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 nº113/2013) for the care and use of laboratory animals. Mice were euthanized when terminally ill, as evidenced by either severe dyspnea or weakness caused leukemic dissemination in the thymus or vital organs (bone marrow, lung, and liver), respectively. For mouse leukemia transplantation assays, 0.5-2 × 10⁶ leukemic cells collected from diseased female TEL-JAK2/TCR-HY, TEL-JAK2, TEL-JAK2/CD3ε−/− or IKL/L mice, were intravenously injected in the tail vein of recipient 8-12-week-old C57BL/6 or NSG mice (for the IKL/L tumor) of the indicated gender and genotype and regularly monitored through peripheral blood detection of leukemic cells (CD45.1+CD45.2+TCR-Vβ6+ TEL-
JAK2/TCR-HY cells or CD4⁺CD8⁺ TEL-JAK2 cells). Anti-CD3ε (145-2C11) mAb or control ChromPure Syrian Hamster IgG (Jackson Immunoresearch) was diluted in sterile PBS and intravenously administered on a regimen of 20 µg/mouse/day for 5 consecutive days for the TEL-JAK2 tumors and on a regimen of 50 µg/mouse/day for 2 periods of 5 consecutive days separated by a 2 days pause for the IκB L/L leukemia. For human leukemia xenotransplantation assays, 1 x 10⁶ fresh leukemic cells obtained from primary NSG mice engrafted with patient-derived cells from T-ALL cases M106, UPNT419, UPNT420, M149, UPNT610, UPNT374 and UPNT525 were intravenously injected in 2-month-old NSG mice. These mice were injected intravenously with either anti-CD3ε OKT3 mAb (BioXCell; 40 µg/mouse/day treatment for 2 rounds of 5 consecutive days separated by a 2 day interval) or the isotype-matched (IgG2a) C1.18.4 mAb (BioXCell), both diluted in PBS. In the curative setting, mice were treated as above when leukemic cells reached 1-4% of blood nucleated cells. Mice were monitored weekly by flow cytometry for leukemic load (FSC hi, hCD7⁺, hCD45⁺ cells) in peripheral blood. Statistical analyses and survival curves were calculated using Prism 5 (GraphPad). Kaplan-Meier survival curves were compared using the log-rank test. Histological analyses were performed on paraffin-embedded sections as detailed in Supplementary methods.

**T-ALL patient samples and cell lines.** Immunophenotypic and oncogenic features of T-ALL patients were identified as described (6, 27, 49, 50) and detailed in Table 1. Fresh or thawed primary T-ALL samples (peripheral blood or bone marrow) obtained at diagnosis from adult and pediatric patients were used, as well as T-ALL cells xenografted in NSG mice. T-ALLs were all included within the GRAALL-2005 study and informed consent was obtained from all patients at
trial entry (trial registration ID: NCT00327678). Studies were conducted in accordance with the Declaration of Helsinki and approved by local and multicenter research ethical committees. All samples used contained ≥ 80% blasts. CD4+CD8+ (DP) human thymocytes were obtained and processed as described in Supplementary methods. The ALL-SIL cell line (DSMZ, Braunschweig, Germany, ACC511) were grown in vitro and transduced as described in Supplementary methods. The ALL-SIL parental line and all derivatives were authenticated by short tandem repeat (STR) DNA profiling on June 10, 2016.

**In vitro TCR stimulation by antigen presenting cells.** Splenocytes were obtained from C57BL/6 female mice, subsequently irradiated (2000 rad) and preincubated with antigenic peptides at 37°C for 1 h (peptide pulsing). Peptides used were DBY (NAGFNSNRANSSRSS), the male antigen epitope specifically recognized by Marilyn transgenic TCR-HY, at 10, 1, 0.1, 0.01, 0.001 and 0.0001 µM and OVA (ISQAVHAAHAEINEAGR), a non-specific peptide, at 10µM. Splenocytes were then plated out in 48-well plates, without removal of peptides. ALL-SIL TCR-HY cells were added to the co-culture system at a 1:100 ratio. Control conditions were performed in parallel with non-pulsed splenocytes, DBY without splenocytes and ALL-SIL without transgenic TCR co-cultured on DBY-pulsed splenocytes. Apoptosis was analyzed by flow cytometry at day 3 of culture, as described in Supplementary methods.

**Flow cytometry analysis of leukemic cells.** Cell suspensions prepared from mouse lymphoid organs, bone marrow or blood were stained with fluorochrome-labelled antibodies, and analyzed on a FACSCalibur cytometer (BD Biosciences), as described (43). The list of antibodies used is described in Supplementary methods. Data was analyzed using Diva, CellQuest (BD Biosciences) and FlowJo (Tree Star) softwares.
Anti CD3/CD28 cell stimulation. Short-term stimulation was performed on cells cultured in serum-free medium for at least 15 min at 37°C prior to stimulation. Murine anti-hCD3 mAb (OKT3, Biolegend) (20µg/ml) was added to the cell culture on ice for 10 min and mAbs were then cross-linked by addition of goat-anti-mouse antibody to a final concentration of 50 µg/ml for 15 min. Stimulation (0-10 min) was triggered by warming cells at 37°C and terminated either by cooling cells in cold PBS or fixing cells for Phosphokinase array and intracellular phosphoprotein (Phosflow) assays, respectively. For prolonged stimulation, a bead-based assay was used in which cell lines were cultured in 96- or 24-well-plates and exposed to 4.5 µm diameter superparamagnetic beads covalently coupled to anti-CD3 and anti-CD28 antibodies (Dynabeads® Human T-Activator CD3/CD28, Invitrogen) (duration of stimulation from 1 to 12 days). Prolonged stimulation of primary T-ALL cells was performed in a co-culture assay with confluent OP9-DL1 in a α-MEM media supplemented with 20% FBS (Hyclone; ThermoFisherScientific), 50 µg/ml streptomycin and 50 IU penicillin and recombinant human cytokines hFLT3-L (5 ng/mL), hIL-7 (2 ng/mL) and hSCF (10 ng/mL) (Miltenyi). Similarly, CD3/CD28 coated-beads were added to the co-culture assay following the manufacturer’s instructions.

Intracellular flow cytometry with phosphorylation-specific antibodies. Stimulation of cells was terminated by fixation with prewarmed formaldehyde (Cytofix Buffer; BD Biosciences) at 37°C for 10min. After cooling on ice for 1 min, cells were pelleted at 4°C and permeabilized in 1 ml of ice-cold methanol (Perm Buffer III; BD Biosciences). After 30 min, cells were washed 3 times with 3ml of PBS and resuspended in 50µl PBS for intracellular staining with Phospho-ERK1/2-Alexa647 (pT202/pY204), Phospho-Stat3-Alexa488 (pY705) (BD Biosciences) or...
Phospho-Akt-Alexa647 (pS473) (Cell Signaling) and incubated for 20 min at room temperature. After washing, cells were resuspended in PBS for flow cytometric analysis on FACSCanto II (BD). Data were processed using Cytobank software.

**Western Blot analyses.** Sample processing and analysis are described in Supplementary methods.

**Flow cytometry analysis of TCR clustering.** ALL-SIL TCR-HY cells were stained with TCR Vβ6-PE mAb (BD Biosciences) for 30 min at 4°C and DAPI for 5 min at room temperature prior stimulation and split into two samples; one sample remained unstimulated while the other one was stimulated by styrene beads (Polyscience) pre-coated with human anti-CD3ε antibody (OKT3, Biolegend) for 10 min at 37°C. Cells were fixed with 1% formaldehyde. Staining was analyzed using ImageStream X mkII (Amnis Merck-Millipore). Data analysis was performed using the IDEAS image analysis software (Amnis).

**Phosphokinase antibody array analysis and pharmacological inhibition.** ALL-SIL, ALL-SIL TCR-HY cells, human DP thymocytes and CD3-negative (UPNT525) and CD3-positive (M149) primary T-ALLs were either left unstimulated or were stimulated with CD3/CD28 mAbs for 5 min. Each stimulated condition was performed in replicate. Cell lysates and Proteome Profiler Human phosphokinase array (R&D Systems) were performed according to the manufacturer's protocol. Chemiluminescence was detected by ChemiDoc XRS+ (Bio-Rad). The average signal (pixel density) of duplicate spots representing each phosphorylated kinase protein was normalized with the average signal of reference positive duplicate spots of each membrane using
Image Lab software (Bio-Rad). Signal ratios for selected phosphoproteins were displayed in a heat-map using Treeview software. Cyclosporine A (Novartis) and PD184352 (Selleckchem) were used at 1µM. Effects were determined by analysis of apoptosis (Annexin V/PI assay) and expression of CD25 and CD69 activation markers.

**Microarray gene expression profiling.** RNA extraction was performed for the following cells: ALL-SIL-TCRαβ-GFP co-cultured on OP9-DL1, ALL-SIL-TCRαβ-GFP without co-culture, ALL-SIL transduced with TLX shRNA (sh-TLX), and ALL-SIL transduced with sh-control vectors. These samples were obtained as previously described (14) in duplicate, and RNA extraction was performed at an early time-point before apoptosis onset (48 h of co-culture for ALL-SIL-TCRαβ-GFP and 48h after puromycin selection for TLX shRNA/sh-control ALL-SIL). RNA hybridization was performed on Affymetrix U133 plus 2.0 microarrays. The statistical data analysis was performed with R version2.9.0 using the “Affy” package from Bioconductor. The probe intensities were log2 transformed and normalized using RMA. Identification of differentially expressed genes was performed by Significance Analysis of Microarrays (SAM), using 500 permutations and a false discovery rate threshold of 5%. Gene Set Enrichment Analysis (GSEA) was performed using the negative selection signature described by Baldwin *et al*, 2007 (14) as gene set. GSEA was run using signal-to-noise for the ranking gene metric and 1000 permutations. All microarray data have been submitted to the Gene Expression Omnibus database under accession numbers: GSE65496.

**Luciferase xenograft studies.** 1x10^6 control pLKO or pLKO-shLAT GFP-Luciferase sorted ALL-SIL TCR-HY cells were intravenously injected into 2 month-old NSG mice (n=10).
Isoflurane-anesthetized mice were intraperitoneally injected with 2.5 mg of D-luciferin (CALIPER Life Sciences) and monitored on a weekly basis to detect luciferase activity using an IVIS Spectrum (Perkin-Elmer). When leukemic cells were detected (22 days post-injection), mice were treated with either OKT3 or control mAb as described above.
Acknowledgments

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REFERENCES


FIGURES LEGENDS

Figure 1. TCR stimulation by antigen presenting cells induces ALL-SIL/TCR-HY cell death. (A) ALL-SIL TCR-HY cells were co-cultured for 3 days with irradiated female splenocytes pulsed with the indicated doses of cognate DBY peptide (top panels), as compared to control conditions (bottom panels): absence of DBY (no peptide), presence of a non-cognate peptide (+ OVA 10 µM), presence of DBY without splenocytes (+ DBY 10 µM) or ALL-SIL parental cells stimulated with splenocytes and DBY. Apoptosis was analyzed by annexin V and propidium iodide (PI) staining. Dot plots were gated on ALL-SIL cells. (B) Graphical representation of apoptosis level in 3 independent experiments as described in A (***P ≤ 0.0001, 0.01 ≤ **P < 0.0001, 0.05 < *P < 0.001).

Figure 2. TCR-HY stimulation prevents T-ALL development. (A) Kaplan-Meier survival curves for male and female TEL-JAK2/TCR-HY mice. (B) Leukemic cell surface immunophenotype of male TEL-JAK2/TCR-HY mice. (C) Flow cytometry analysis of TCRβ and TCR-Vβ6 surface expression on leukemic cells from three males and three female TEL-JAK2/TCR-HY mice that developed T-ALL. (D) Kaplan-Meier survival curves for secondary female and male recipient syngeneic mice transplanted with female TEL-JAK2/TCR-HY leukemic cells. (E) Spleens from recipient female and male mice transplanted as described in D but sacrificed 17 days later. (F) Leukemic burden analysis of spleen (left) and bone marrow (right) of mice transplanted as in D but sacrificed at the time females became terminally ill. (G) Kaplan-Meier survival curves of Swiss Nude allogenic mice transplanted with female TEL-JAK2/TCR-HY leukemic cells (ns: non-significant). (H) Kaplan-Meier T-ALL-free survival curves of Rag2−/− syngeneic mice transplanted with female TEL-JAK2/TCR-HY leukemic cells.
Figure 3. Anti-CD3 stimulation of ALL-SIL/TCR-HY cells induces TCR signaling and apoptosis. (A) Apoptosis detection by annexin V and PI staining of ALL-SIL and ALL-SIL TCR-HY cells stimulated by anti-CD3/CD28 coated-beads at the indicated time points. (B) Percentage of annexin V-positive ALL-SIL or ALL-SIL/TCR-HY cells either left unstimulated or stimulated by anti-CD3/CD28 coated-beads for the indicated time. (C) Western Blot analysis of caspase 3 and caspase 7 cleavage in ALL-SIL and ALL-SIL/TCR-HY cells either left unstimulated or stimulated by anti-CD3/CD28 coated-beads for 48 and 72 h. Actin was used as a loading control. (D) Growth curve of unstimulated and anti-CD3/CD28-stimulated ALL-SIL and ALL-SIL/TCR-HY cells. (E) Percentage of unstimulated or anti-CD3/CD28-stimulated ALL-SIL/TCR-HY cells in S-phase of the cell cycle as measured by EdU incorporation and 7-AAD staining of DNA. (F) ImageStream flow cytometry imaging of ALL-SIL/TCR-HY single cells showing the recruitment of TCR-Vβ6 to a contacting anti-CD3 coated-bead (arrowhead) (each row shows one cell in brightfield, phycoerytrin and DAPI fluorescence and merge of 3 channels, ×40). (G) Heat-map representation of normalized ratio of intensity of indicated phosphorylated proteins in Proteome Profiler Human phosphokininase array, representative of 2 experiments. (H) Phospho-ERK, phospho-AKT and phospho-STAT3 expression detected by flow cytometry in non-stimulated (top line in each panel) or 30 min anti-CD3/CD28-stimulated (bottom line) ALL-SIL/TCR-HY cells. (I) Percentage of annexin V-positive unstimulated (left) or anti-CD3/CD28-stimulated (right) ALL-SIL/TCR-HY cells at day 5, in the presence of indicated signaling pathway inhibitors. (J) Inhibitory effects of indicated drugs on expression of T-cell activation markers (CD25 and CD69) in anti-CD3/CD28-stimulated ALL-SIL/TCR-HY for 2 days.
Figure 4. Anti-CD3 induces human primary T-ALL apoptosis in vitro and mimics thymic negative selection. (A) Apoptosis analysis of primary T-ALL cells (UPNT419) co-cultured on OP9-DL1 cells with or without anti-CD3/CD28 stimulation for 2 or 4 days. (B) Flow cytometry analysis of the induction kinetics of ERK1/2 phosphorylation (right) upon anti-CD3 (OKT3) stimulation of surface TCR/CD3-positive primary T-ALL (UPNT419) (left). (C) Box and Whiskers plots presentation of apoptosis induction for each individual TCR/CD3-positive primary, diagnostic T-ALL cells (24 cases) as compared to apoptosis induction for each individual TCR/CD3-negative primary T-ALL cells (12 cases) after 3 days of co-culture on OP9-DL1 in the presence or absence of CD3/CD28 stimulation (P** < 0.0001, ns: non-significant). (D) Before and After plot representation of the comparison of apoptosis induction in TCR/CD3-positive primary, diagnostic T-ALL cells (24 cases) versus TCR/CD3-negative primary T-ALL cells (12 cases) as described in C (P** < 0.0001 , ns: non-significant). (E) Gene Set Enrichment Analysis of the negative selection gene signature (14) comparing the ALL-SIL transcriptome with or without TCR activation, showing an enrichment in the TCR activation transcriptome (FRD, false discovery rate). (F) Scatter plot showing up- and down-regulated genes in response to TCR activation in ALL-SIL cells. The negative clonal deletion gene expression signature is detailed.

Figure 5. TCR stimulation by agonistic monoclonal antibody in vivo administration inhibits mouse T-ALL development. (A and B) Percentage of TEL-JAK2/TCR-HY leukemic cells in peripheral blood (PB) (A) and Kaplan-Meier survival curves (B) of female mice treated daily as indicated (arrows) with hamster anti-CD3 mAb or control IgG. (C) Percentage of TEL-JAK2 leukemic cells (expressing endogenous TCR) in peripheral blood leukocytes of mice that were treated daily with anti-CD3 or control hamster IgG. (D) Kaplan-Meier survival curves of mice
injected with either TEL-JAK2 leukemic cells (expressing endogenous TCR), or TEL-JAK2/CD3ε−/− leukemic cells and treated daily with anti-CD3 or control hamster IgG. (E) Percentage of IKL/L leukemic cells in peripheral blood leukocytes defined as FSC^high, Thy1.2^CD25^+ in control IgG or anti-CD3-treated NSG mice at the indicated time point. (F) Kaplan-Meier survival curves of NSG mice injected with IKL/L leukemic cells treated with hamster anti-CD3 mAb or control IgG as indicated.

Figure 6. TCR stimulation by in vivo administration of agonistic monoclonal antibody in a preventive setting inhibits human T-ALL development. (A) Flow cytometry monitoring of leukemia burden in peripheral blood from NSG mice injected with human T-ALL M106 and UPNT419 and treated daily with either OKT3 or an isotype control IgG (arrows). (B) Tumor burden in peripheral blood of NSG mice injected with human primary T-ALL cells either mCD3/TCR positive: M106 (n=6 in each group), UPNT419 (n=5 in each group), UPNT420 (n=5 in each group), M149 (n=6 in each group) UPNT610 (n=5 in each group), or mCD3/TCR negative: UPNT525 (n=5 in each group), or heterogeneous for CD3/TCR expression: UPNT374 (n=5 in each group). Mice were treated with either control IgG or OKT3 in a preventive setting. Data show tumor burden in peripheral blood analyzed at the time when IgG-treated mice were moribund and sacrificed, except for UPTN610. For all TCR+ leukemias the p value was <0.0001 (C) Leukemia burden in spleen (top panel) and BM (bottom panel) of control and OKT3-treated mice that were sacrificed when IgG-treated control mice were leukemic (day 31 of Fig. 4A). (D) H&E-stained bone marrow (BM) and liver sections of mice analyzed in B. Arrow indicate infiltrating cells in liver perivascular spaces and sinusoids. (E) Kaplan-Meier survival curves of NSG mice transplanted with M106 and UPNT419 human T-ALL cells analyzed as in A. (F) Flow
cytometry analysis of TCR cell surface in leukemic BM cells of NSG mice xenografted with T-ALL UPNT374 treated with either control IgG (black/grey tracings) or OKT3 (red to pink tracings) at the time of sacrifice. Green tracing shows staining with the control isotypic antibody.

Figure 7. TCR stimulation by administration of agonistic monoclonal antibody in a curative setting inhibits human T-ALL development.

(A) Tumor burden in NSG mice xenotransplanted with human ALL-SIL/TCR-HY luciferase-expressing cells transduced with either the LKO control vector (top panels) or the LKO vector encoding LAT shRNA #1 (bottom panels). Once leukemic, mice daily received either IgG control or anti-CD3 mAb (OKT3), and bioluminescence at day 48 post-xenotransplantation is shown (Luminescence color scale: minimum = 8.00 x 10^4 to maximum = 2.00 x 10^6 radiance). (B) Bioluminescence quantification over time of the experiment shown in A. (C) Tumor burden in peripheral blood of NSG mice xenotransplanted with human T-ALL cells (UPNT419) and treated with either control IgG or OKT3 when mice were leukemic. (D) Kaplan-Meier survival curves of NSG mice transplanted with UPNT419 analyzed in C.
Figure 1

A

ALL-SIL TCR-HY + splenocytes + DBY

+ 0.0001µM  + 0.001µM  + 0.01µM  + 0.1 µM  + 1µM

Annexin V

B

Percentage of Annexin V positive cells

Concentration of DBY peptide pulsed on splenocytes
Figure 3

A. CD3/CD28 stimulation

Day 3

ALL-SIL

211
64
3

ALL-SIL TCR-HY

5120
71
4

Day 5

14
64
2

5137
53
5

Day 7

10
16
1

1613
35
1

B. % Annexin V positive cells

Days

0 1 3 5 7

No stimulation

+CD3/CD28

ALL-SIL

+ALL-SIL TCR-HY

C. Caspase 3

Cleaved caspase 3

Cleaved caspase 7

D. Absolute cell number (x10^4)

Days

0 5 10

No stimulation

+CD3/CD28

ALL-SIL

+ALL-SIL TCR-HY

E. Edu positive S phase cells (%)

Days

0 3 5

No stimulation

+CD3/CD28

F. Brightfield

TCR Vβ

DAPI

Merge

G. p-ERK1/2, p-Akt, p-Stat3

No stimulation

+CD3/CD28

H. % Annexin V positive cells

+No stimulation

+CD3/CD28

I. CD25

CD69

J. DMSO

PD184352

Cyclosporine A

1µM DMSO

1µM PD184352

1µM cyclosporine A

1µM cyclosporine A + 1µM PD184352
Primary T-ALL unstimulated

Primary T-ALL +CD3/CD28

Day 2

Day 4

Annexin V

Percentage of Annexin V positive cells

No stimulation +CD3/CD28

No stimulation +CD3/CD28

T-ALL mCD3+ (n=24)

T-ALL mCD3- (n=12)

Log2(expression level) in «no TCR activation»

Downregulated genes in TCR activation

Upregulated genes in TCR activation

Negative selection signature

Log2(expression level) in «TCR activation»

FDR < 0.0001

Enrichment score (ES)

CD3

TCRαβ

p-ERK

T-ALL mCD3+ (n=24)

T-ALL mCD3- (n=12)
Figure 5

A. TEL-JAK2/TCR-HY
- % leukemic cells in PB (CD45.1+CD45.2+) CD8+V(6+)
- Time (days)

B. TEL-JAK2/TCR-HY
- T-ALL free survival (%)
- Time (days)

C. TEL-JAK2
- % leukemic cells in PB (CD4+CD8+)
- Time (days)

D. TEL-JAK2 and TEL-JAK2/CD3ε−/
- T-ALL free survival (%)
- Time (days)

E. IKL/L T-ALL
- % of leukemic cells in PB (large Thy1.2+, CD25+)
- Time (days)

F. IKL/L T-ALL
- T-ALL free survival (%)
- Time (days)
Figure 6

A. Graph showing the percentage of leukemic cells in PB (hCD45+ hCD7+) for M106 and UPNT419 over time (days) with IgG and OKT3. The graphs illustrate the progression of leukemic cells with IgG and OKT3 treatment over time.

B. Bar graph showing the % of leukemic cells in PB (hCD45+ hCD7+) for M106, UPNT419, UPNT420, M149, M1610, UPNT525, and UPNT374. The bars represent IgG and OKT3 treatments, with IgG in black and OKT3 in pink.

C. Graph showing the % of leukemic cells in spleen (hCD45+ hCD7+) for IgG and OKT3 at Day 31. The graph indicates a significant difference between IgG and OKT3 treatments.

D. Images of the sternum and liver from Day 31, showing the distribution of leukemic cells with IgG and OKT3 treatments.

E. Graph showing T-ALL-free survival for M106 with IgG and OKT3 treatments. The graph shows a significant difference between IgG and OKT3 treatments with a p-value of 0.029.

F. Graph showing T-ALL-free survival for UPNT419 with IgG and OKT3 treatments. The graph shows a significant difference between IgG and OKT3 treatments with a p-value of 0.025.

Legend:
- IgG: Black
- OKT3: Pink
- mCD3+TCR+: Black bars
- mCD3-TCR-: Pink bars
- Heterogeneous TCR expression: Pink bars
Figure 7

A

IgG-Day48

OKT3-Day48

SIL-ALL pLKO

SIL-ALL shLAT

B

Time (days)

photon/sec

0.0

5.0 × 10^7

1.0 × 10^8

1.5 × 10^8

Ctrl IgG (n=5)

Ctrl OKT3 (n=5)

shLAT IgG (n=5)

shLAT OKT3 (n=5)

C

% leukemic cells in PB

nCD45+ nCD7+

0 20 40 60 80 100

IgG (n=3)

OKT3 (n=4)

D

T-ALL free survival (%)

0 50 100

IgG (n=3)

OKT3 (n=4)
Triggers the TCR developmental checkpoint activates a therapeutically targetable tumor suppressive pathway in T-cell leukemia

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