Supplementary methods, Figure and Table

SUPPLEMENTARY METHODS

Histopathology. Organs were harvested from mice and immersion-fixed in 50% ethanol, 10% formalin, 5% acetic acid solution for 2 h at room temperature. After dehydration in increasing concentrations of ethanol, organs were embedded in paraffin. 7-µm paraffin sections were stained with hematoxilin and eosin following standard procedures. Images were obtained using a microscope (DMRB; Leica) equipped with a digital camera (DXC-950P; Sony) and TRIBVN image software.

Human DP thymocytes. Normal thymus samples were obtained from children undergoing cardiac surgery at the Necker-Enfants-Malades Hospital, with informed consent from the parents. Thymi were mechanically disrupted on a 75µm nylon cell strainer. Cells were washed twice with cold PBS. To assess the percentage of DP thymocytes, staining with a cocktail of CD4, CD8, TCRαβ, CD3 and CD45 mAbs was detected using a FACS CantoII (BD Biosciences). Samples with more than 80% DP thymocytes were selected.

Cell lines. The ALL-SIL cell line (DSMZ, Braunschweig, Germany, ACC511) was transduced with a VSV-G pseudotyped lentiviral vector carrying a tricistronic TCRα-TCRβ-GFP vector (1) or the codon-optimized sequence of murine class II MHC-restricted (Vα1.1Vβ6) TCR-HY (2) or encoding both GFP and Firefly Luciferase (3). Cells expressing either Vβ6 TCR or GFP were sorted by flow cytometry. Cells were cultured in RPMI-1640 medium supplemented with 50 µg/ml streptomycin, 50 IU penicillin, and 20% fetal bovine

serum (Gibco, Life Technology). To knock-down LAT expression in SIL-ALL/TCR-HY luciferase-expressing cells, these were transduced with either LKO control lentivirus or LKO derivatives encoding two distinct shRNA to LAT (TRCN0000002934 (#1); TRCN0000002935 (#2); GE-Dharmacon) and selected with 0.4μg/ml of puromycin. OP9 and OP9-DL1 cells were cultured in α-MEM medium supplemented with 20% FBS (Hyclone, Thermo Fisher Scientific), 50 μg/ml streptomycin and 50 IU penicillin. All cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. For co-culture assays with ALL-SIL cells, confluent OP9 or OP9-DL1 were used in a α-MEM media supplemented with 20% FBS (Hyclone; Thermo Fisher Scientific), 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), with and without transwell (Millicell, Millipore).

Flow cytometry analysis of leukemic cells. FITC-, PE-, PE-Cy5-, PerCP/Cy5.5- or APC-conjugated antibodies specific for murine CD4 (H129.19), CD8α (53-6.7), CD3ε (145-2C11), TCRβ (H57-597), Vβ6 TCR (RR4-7), CD25 (7D4), CD24 (M1/69), B220 (RA3-6B2), CD19 (1D3), IgM (R6-60.2), IgD (11-26c.2a), Ig kappa (187.1), CD45.1 (A20), and CD45.2 (104) were from Biolegend and BD Biosciences. Human T-ALL cells were stained with: CD1a-APC (HI149), sCD3-Alexa700 (UCHT1), CD4-V450 (RPA-T4), CD5-PerCP-Cy5.5 (L17F12), CD8-PerCP-Cy5.5 (SK1), CD25-PE-Cy7 (2A3) and CD69-V450 (FN50) (all from BD Biosciences); CD7-APC (124-1D1), CD7-FITC (4H9) and CD45-APC (2D4) (eBioscience); TCRαβ-PE (IP26A) and TCRγδ-FITC (Beckman-Coulter).

Apoptosis, proliferation and cell cycle analysis. Apoptosis was analyzed by flow cytometry at different culture time-points, using annexinV-APC and propidium iodide (PI) (BD Biosciences) following the manufacturer's instructions. Absolute cell counts were determined

using Perfect Count Microspheres (Cytognos) by flow cytometry. Cell cycle was assayed by flow cytometry using Click-iT® EdU Pacific Blue™/SYTOX® AADvanced staining (Invitrogen), following the manufacturer's instructions. Data were analyzed using DIVA software (BD Biosciences).

Western Blot analyses. Cells were washed in cold PBS and lysed in 50mM Hepes pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1% Triton, 10% glycerol, 1.5 mM $MgCl_2$supplemented with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, ThermoScientific). Proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). Immunoblotting was performed with Caspase3, Caspase7 (BD Biosciences), and Actin (Abcam) primary antibodies and bovine anti-mouse_IgG-HRP (Santa Cruz Biotechnology) secondary antibody. Chemiluminescence was detected by ChemiDoc XRS+ (Bio-Rad) using the West Dura SuperSignal kit (ThermoScientific).

References

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Supplementary Table 1. Immunophenotypic and oncogenic characteristics of T-ALL samples.

GL, Germ Line; HD, Heterodimerization Domain of NOTCH1; PEST, proline glutamate serine threonine domain of NOTCH1; * indicates which of these T-ALL primary cells were used in xenograft experiments.

Supplementary Figure 1. Anti-CD3 stimulation of primary human T-ALLs. (A) Heat-map representation of normalized ratio of signal intensity of the indicated phosphorylated proteins (Proteome Profiler Human phosphokinase array) between CD3/CD28–stimulated cells *versus* control IgG in CD3-negative (UPNT525) and CD3-positive (M149) primary T-ALLs. (B) Phospho-ERK, phospho-AKT and phospho-STAT3 expression detected by flow cytometry in non-stimulated (grey line) or anti-CD3/CD28-stimulated (red and blue lines) primary T-ALLs. (C) Percentage of Annexin V-positive CD3-positive primary T-ALL cells either unstimulated (left) or anti-CD3/CD28-stimulated (right) at day 3, in the presence or absence of the indicated signaling pathway inhibitors.

Supplementary Figure 2. TCR stimulation by *in vivo* administration of agonistic monoclonal antibody in a preventive setting inhibits human TCR+ T-ALL development. Kaplan-Meier survival curves of NSG mice transplanted with primary T-ALL cells being either TCR+ (M149 and UPNT420), TCR-negative (UPNT525) or showing an heterogeneous expression of TCR (UPNT525) and treated with the control IgG or anti-CD3 as in Figure 6.

Supplementary Figure 3. TCR signaling is essential to the anti-leukemic effect of OKT3 (A) Western blot analysis of LAT expression in LKO-transduced control ALL-SIL/TCR-HY

cells as compared to cells expressing two independent shRNA against LAT. STAT5 is used as a loading control (bottom panel). (B) Flow cytometry analysis of TCR-HY cell surface of cells described in A. Black tracing: control cells, grey tracing shLAT cells, green tracing shows cells stained with isotypic control antibody. **(C)** Apoptosis detection by annexin V and PI staining of cells described in A and of the non-transduced ALL-SILL/TCR-HY parental cells (par.), 5 days after stimulation by anti-CD3/CD28 coated-beads. **(D)** Flow cytometry monitoring of leukemia burden in peripheral blood from NSG mice injected with ALL-SIL/TCR-HY cells and transduced with either control (LKO) or LKO shRNA LAT #1 (shLAT) vectors, and daily treated with either OKT3 or an isotype control IgG as indicated by arrows. **(E)** Leukemia burden in BM of the OKT3-treated and control IgG-treated mice described in D, sacrificed when IgG-treated control mice were terminally ill (day 48). **(F)** Western blot analysis of LAT expression in BM leukemic cells retrieved from mice sacrificed in E. Flow-cytometry sorted shLAT-leukemic cells (BM) obtained from 2 control IgG-treated mice and 2 OKT3-treated mice were compared for LAT expression to LKO-control leukemic cells obtained from control IgG-treated mice.