Supplementary material and methods

Purification of primary mononuclear cells from healthy donors and patients

Peripheral blood of three healthy donors was obtained by venipuncture and immediately processed to recover the *buffy coats*, further used for the isolation of peripheral blood mononuclear cells (PBMC) by density gradient centrifugation on Ficoll-Paque™ Plus (density: 1.077 g/mL, 17144003, GE Healthcare, Italy). Briefly, 4 mL of *buffy coat* were placed on 3 mL Ficoll-Paque™ Plus. After centrifugation (600 × g, 40 minutes, 15 °C), the white mononuclear cell layer at the interphase was washed twice with PBS (10 mL, 600 × g, 10 minutes, 15 °C). Pellets were suspended in RPMI-1640 medium (ECB9006L, Euroclone, Italy) completed with 10% heat inactivated Fetal Bovine Serum (FBS-HI, F4135, Sigma-Aldrich, Italy) and further used for T-lymphocytes ($CD3^+$) purification.

Bone marrow aspirates were collected at first onset or relapse as part of diagnostic procedures, and used for research purpose only when clinical procedures had been completed. Patients' bone marrow aspirates (~3–5 mL) were diluted with PBS to a final volume of 10 mL and loaded on Ficoll-Paque™ Plus (5 mL); after centrifugation (800 × g, 20 minutes 15 °C) interphase was recovered and washed twice with PBS (7 mL, 300 xg, 10 minutes, 15 °C). Pellets containing mononuclear cells were suspended in serum-free X-VIVO™ 15 (04-418Q, Lonza, Italy) supplemented with gentamicin 0.1 mM (G1397, Sigma-Aldrich, Italy) at the final concentration of 3 × 10^6 cells/mL, and used for the cytofluorimetric *in vitro* assay.

Primary $CD3^+$ cells purification

T-lymphocytes ($CD3^+$) were purified starting from 50x10^6 PBMC using the human Pan T Cell Isolation Kit (130-096-535, Miltenyi Biotec, Italy), and separated on magnetic columns in a VarioMACS Separator (130-090-282, Miltenyi Biotec, Italy), according to manufacturer's instructions; mean cell recovery was 30.26 × 10^6 ± 1.61 × 10^6. T-lymphocytes purification was verified by cytofluorimetric analysis using MACSQuant 10 (Miltenyi Biotec, Italy) and the viability was determined by trypan blue exclusion assay; aliquots (5–6 × 10^6 cell/mL, mean ± SD $CD3^+$: 80.75 ± 13.8%) were frozen in FBS-10% DMSO (D2650, Sigma-Aldrich, Italy) and kept at –80 °C.

Cell cultures

NALM6 and REH cells were cultured in standard complete RPMI-1640 medium supplemented with 10% heat inactivated FBS, 2 mM L-glutamine (ECB3000D-20, Euroclone, Italy), 1X Penicillin-Streptomycin solution (P0781, Sigma-Aldrich, Italia) or in in serum-free X-VIVO™ 15 (04-418Q, Lonza, Italy) plus gentamycin 0.1 mM, according to experimental needs. Cell passages were performed according to standard procedures.

Aliquots of isolated T-lymphocytes ($CD3^+$) were thawed quickly using pre-warmed (37 °C) complete RPMI-1640 medium. After centrifugation (300 × g, 5 minutes, 15 °C), cells were washed three times (3 mL PBS, 300 xg, 5 minutes, 15 °C) and kept for 24 hours at a concentration of 1 × 10^6 cell/mL in complete RPMI-1640 medium supplemented with 10 ng/mL human IL-2 in a 24-wells plate, before being switched to X-VIVO™ 15- gentamicin 0.1 mM ± IL-2 as indicated in each experiment.

All cell cultures were maintained in a humidified incubator at 37 °C with 5% CO_2, according to

standard procedures.

Western Blot

10×10^6 B-cells (NALM6, REH) were lysed on ice with 100 µL of lysis buffer (10 mM Tris-HCl pH 7.4, 0.1% SDS, 100 mM NaCl, 100 mM EDTA, Halt™ Protease Inhibitor Cocktail 1x (87786, Thermo Fisher Scientific, Italy), by 10 seconds sonication for 3 times (Ultrasonicator UP50H Hielscher Ultrasound Technology, Imlab, France). After centrifugation (16,000 × g, 10 minutes, 4 °C), supernatants were aliquoted and stored at –80 °C. Protein concentrations were determined by Bradford analysis (B6916, Sigma-Aldrich, Italy), according to manufacturer's instructions; absorbance was measured at 570 nm on a Microplate Reader EL311 (214891, BioTek Instruments, Inc).

Whole cell extracts (25 µg) were fractionated by SDS-PAGE on Bolt™ 10% Bis-Tris Plus Gels (NW00102BOX, Thermo Fisher Scientific, Italy) at 180 V, 150 mA, 30–40 minutes, and then gels were blotted to nitrocellulose membranes (PB7320, Thermo Fischer Scientific, Italy) at 25 V, 2.5 A for 15 minutes. After blocking with 5% nonfat milk in Tween/Tris-buffered salt solution (T-TBS, 50 mM Tris-Cl, 150 mM NaCl, pH 7.5, 0.1% Tween 20) for 1 hour, membranes were incubated overnight at 4 °C with rabbit primary antibodies (anti human PAX5 (GTX89903, GeneTex, Italy) and ant-human Vinculin (ab129002, Abcam, Italy), diluted 1:3000 or 1:10000 in blocking solution, respectively). Membranes were washed three times with T-TBS for 5 min and further incubated for 1 hour at 4 °C with horseradish peroxidase-conjugated anti-rabbit antibody (AP132P, Merck, Italy) and anti-goat antibody (ab6885, Abcam, Italy) diluted 1:10000 in T-TBS 5% nonfat milk. Membranes were incubated with LiteAblot TURBO Extra Sensitive Chemiluminescent Substrate (EMP012001 Euroclone). Exposure of membrane was made on light-sensitive film (Z373508-Carestream® BioMax® light film, Sigma-Aldrich). For densitometry analysis of PAX5 (Figure S1), two lysates per cell line were considered; bands of interest were quantified with ImageJ software, after normalizing with vinculin.

Real-time PCR analysis for PAX5 and CD19

Total RNA was extracted from REH, NALM6 and ALL-SIL cell lines. The T-cell leukemia cell line ALL-SIL ($CD3^+/CD19^-$; ACC 511, DSMZ Germany) was included as negative control. Total RNA was extracted using TRIzol reagent (15596018, Invitrogen, ThermoFisher Scientific, Italy) according to the manufacturer's instructions. The RNA was quantified using Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Italy). RNA was reversed-transcribed into cDNA using the High-Capacity RNA-to-cDNA kit (4387406, Applied Biosystem, ThermoFisher Scientific, Italy). Expression of *PAX5* and *CD19* was assessed 3 times in triplicate by Real-time PCR using the KiCqStart SYBR Green qPCR Ready Mix (KCQS00, Sigma-Aldrich, Italy). Pre-designed primer sequences (KSPQ12012, Sigma-Aldrich, Italy) are shown in **Supplementary Table 1**. Thermal cycling conditions were 40 cycle at 5 seconds at 95 °C and 30 seconds at 60 °C, after an initial step of 30 seconds at 98 °C (Thermal Cycle Dice Real Time System, BIO-RAD, Italy). Relative quantification is represented with respect to the housekeeping gene beta-actin (ACTB).

Trypan blue exclusion assay

Cell viability was determined by trypan blue exclusion assay; experiments were performed three times. NALM6 and REH cells were seeded in 96-wells plates at different initial concentrations

(ranging from 62,500-500,000 cells/mL) either in complete RPMI-1640 medium or in serum-free X-VIVO™ 15 plus gentamycin 0.1 mM. T-lymphocytes were seeded in 24-wells plates at 84,000 cells/mL in X-VIVO™ 15 supplemented with different concentrations of IL-2 (0, 10, 20, 100 ng/mL). The number of viable cells was manually counted by a single operator on aliquots of cell suspensions added with 0.04% trypan blue (T8154, Sigma-Aldrich, Italy) loaded into a BRAND® counting chamber BLAUBRAND® with Bürker pattern (BR718920 Sigma-Aldrich, Italy). Cells that stained blue were considered as nonviable.

Supplementary Results

CD19$^+$ NALM6 or REH, but not T-lymphocytes, can proliferate in serum-free medium

We hypothesized that non-standard serum-free medium X-VIVO™ 15 should be preferred to conventional 10% FBS-supplemented RPMI-1640 for culturing B-cells (CD19$^+$) and T cells (CD3$^+$) in the blinatumomab *in vitro* cytofluorimetric assay. In particular, X-VIVO™ 15 could avoid experimental concerns related to the use of FBS in immunological assays (e.g.,: non-standardized and undefined mixture composition, animal origin, presence of serum peptidases and proteinases able to degrade blinatumomab). Moreover, X-VIVO™ 15 is an optimized medium to expand all T cell subsets in the absence of human serum. In the cytofluorimetric assay, CD3$^+$-cells were not further separated in CD4$^+$ and CD8$^+$ lymphocytes since both types are capable of blinatumomab-induced cytotoxicity, although CD8$^+$ cells showed a greater and quick cytotoxic capability than other activated cells, with specific production of cytotoxic factors and cytokines. The use of X-VIVO™ 15 could thus guarantee a better control of T-cells responsiveness and a more consistent inter-experiments performance.

To assess the effects of medium switching on B-ALL cell lines growth, NALM6 and REH were cultured at different seeding concentrations (ranging from 62,500-500,000 cells/mL) in standard 10% FBS supplemented RPMI-1640 and in serum-free X-VIVO™ 15; the increase in cell number was monitored daily over a 7 day-period by trypan blue exclusion test. For both cell lines, exponential growth curves in the two media do not differ at any time and at any initial concentration (RPMI-10% FBS *versus* X-VIVO™ 15, two-way ANOVA, Bonferroni correction, $p > 0.05$, figure not shown). When cultured at low concentration in X-VIVO™ 15 (i.e., 62,500 and 125,000 cell/mL, Figure S2A and S2B, respectively), the number of REH cells become significantly lower than the number of NALM6 after day +6 (two-way ANOVA, Bonferroni post-test, $p < 0.05$). Growth curves do not differ over the time period considered at the higher concentrations (i.e., 250,000 and 500,000 cells/mL, Figure S2C and S2D, respectively); however, when 500,000 cells/mL suspension was kept longer than 4 days, both cell lines begin to die. A seeding concentration of 250,000 B-cells/mL in 96-wells plate (initial 50,000 cells/well) was thus chosen for the blinatumomab *in vitro* assay, to guarantee a similar number of alive growing NALM6 or REH cells in the untreated condition for one week.

Cell viability of T-lymphocytes in serum-free X-VIVO™ 15 (initial concentration: 84,000 cell/mL) was also measured on a daily basis by trypan blue exclusion assay. In absence of IL-2, a cytokine that regulates the activities of leucocytes, cell death begins since the first day (survival mean ± standard deviation (SD): 74.93 ± 15.55%, n = 3) and is almost complete after a week (12.48 ± 1.04% at day +7). Supplements of IL-2 in culture medium do not improve the lymphocytes

viability at any time point (no IL-2 *versus* IL-2 (any concentration), two-way ANOVA, Bonferroni correction, $p > 0.05$, Fig. S3). The lowest concentration of IL-2 was thus chosen for the blinatumomab *in vitro* assay.

Supplementary Tables and figures:

Table 1. Pre-designed primer sequences for real-time PCR. *ACTB*, beta-actin; *PAX5*, Paired Box 5*; CD19*, Cluster of Differentiation 19.

Gene	Primer	Sequence 5'->3'
ACTB	Forward	GACGACATGGAGAAAATCTG
	Reverse	ATGATCTGGGTCATCTTCTC
PAX5	Forward	CTCTGCAAACCAATAGACTG
	Reverse	ATGGATGGATAGTCAGACAG
CD19	Forward	GATGCAGACTCTTATGAGAAC
	Reverse	TCAGATTTCAGAGTCAGGTG

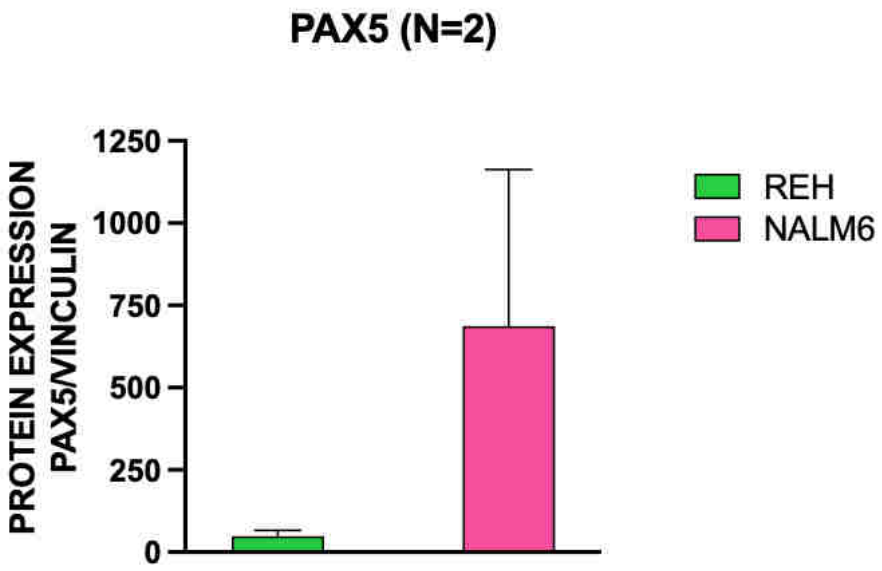

Fig. 1. Protein quantification of PAX5 in B-ALL cell lines. The PAX5 levels were normalized to vinculin after western blot analysis (n = 2).

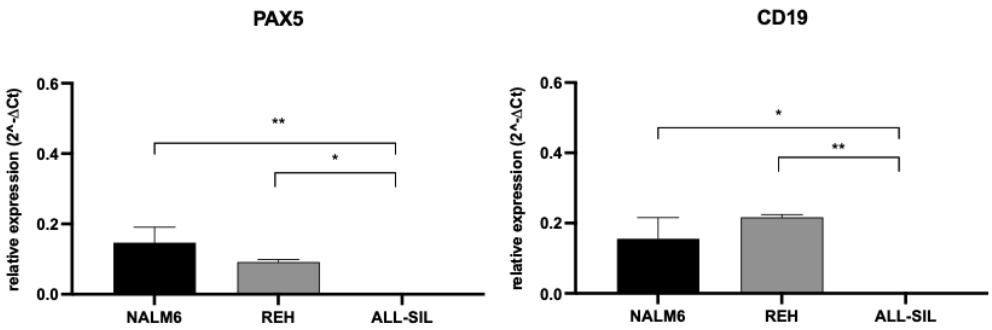

Fig. 2. Real-time PCR analysis of *PAX5* and *CD19* genes. Data are reported as mean ± SD (n = 3). * *p*-value < 0.05, ** *p*-value < 0.01, one-way ANOVA. ALL-SIL is a T-cell leukemia cell line ($CD3^+/CD19^-$) included in the analysis as negative control.

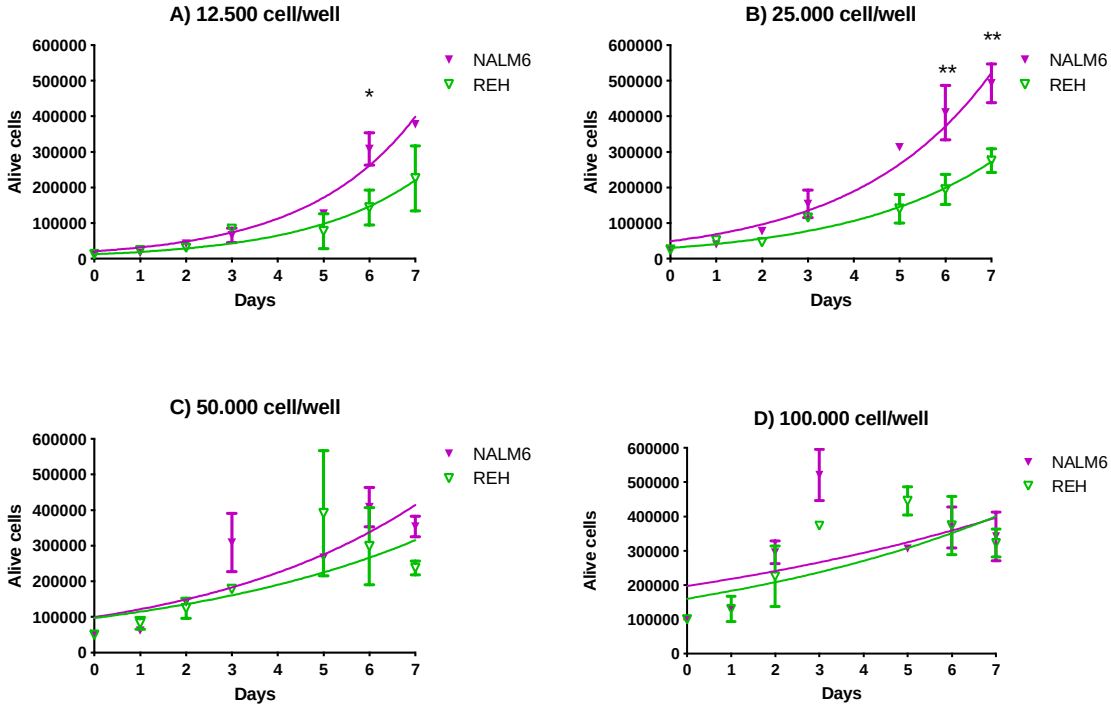

Fig. 3. NALM6 and REH growth curve in X-VIVO™-15 over a 7 day-period and at different seeding concentrations. (A) 0.0625 × 10^6 cells/mL, (B) 0.125 × 10^6 cells/mL, (C) 0.25 × 10^6 cells/mL, (D) 0.5 × 10^6 cells/mL. *p < 0.05, **p < 0.001, two-way ANOVA, Bonferroni post-test.

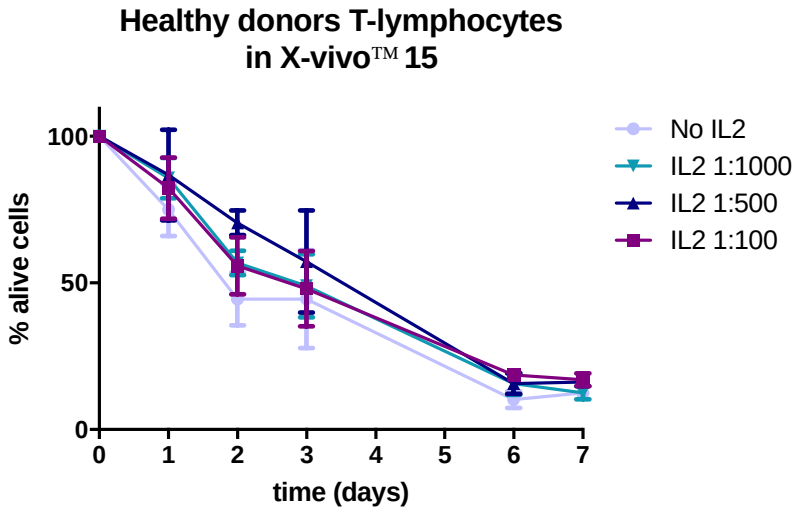

Fig. 4. Healthy donors CD3⁺ T-lymphocytes viability in X-VIVO™ 15 over a 7 day-period at different IL-2 concentrations.

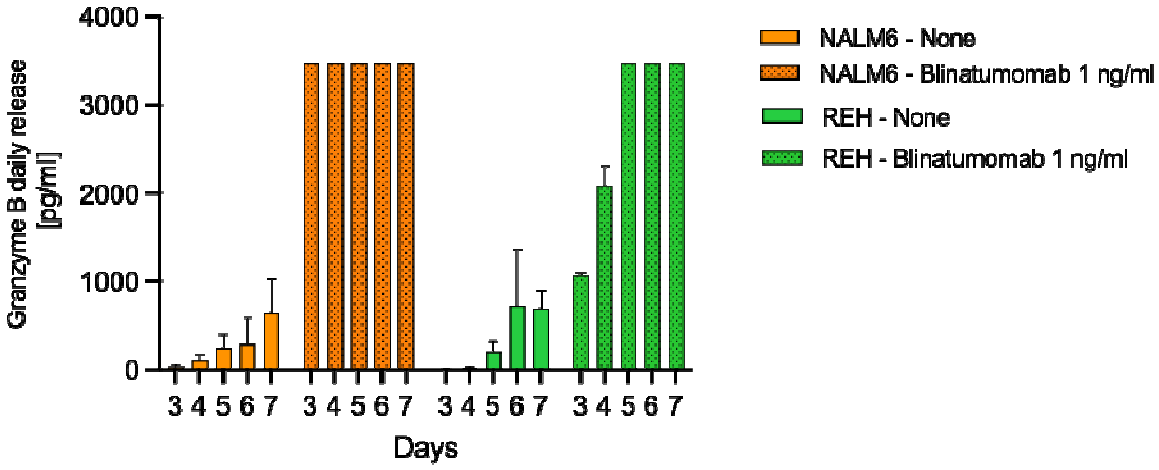

Fig. 5. Granzyme B daily release in CD3⁺: CD19⁺ co-cultures (initial effector-to-target seeding ratio, 1:10) at indicated days.