A novel CD34-specific T-cell engager efficiently depletes acute myeloid leukemia and leukemic stem cells *in vitro* and *in vivo*

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Received: June 23, 2021.
Accepted: February 1, 2022.
Prepublished: February 10, 2022.
https://doi.org/10.3324/haematol.2021.279486

SUPPLEMENTARY DATA

Expression and purification of BiTE and controls

Monoclonal anti-CD34 antibody (αCD34), CD34/CD3 and RSV/CD3 BITEs were constructed by recombinant DNA technology and purified from the supernatants of transfected Chinese hamster ovary cells following standard procedures. All antibodies were produced by Evitria AG (Switzerland). Briefly, corresponding cDNAs were cloned into Evitria's vector system using conventional (non-PCR based) cloning techniques. The seed was grown in eviGrow (Evitria) medium, transfected with eviFect (Evitria), and grown after transfection in eviMake (Evitria). Supernatant was harvested by centrifugation and subsequently filtrated (0.2 μm). αCD34 was generated based on the hMy10 IgG clone, raised against the $hCD34^+$ cell line KG1a, and purified using MabSelect^{TM} SuRe^{TM} (Cytiva, GE Healthcare Life Sciences). CD34-specific BiTE was generated by fusing the anti-hCD34 single chain variable fragment (scFv) from hMy10 clone to the heavy chain of the anti-hCD3 antibody clone L2K-07, a humanized CD3ε-specific antibody derived from blinatumomab, using a $(Gly_4Ser)_1$ linker. RSV-specific BiTE was generated by fusing the anti-RSV.F scFv from clone B21M to the same anti-hCD3 ScFv clone cited above. The BITEs design schematics are detailed in **Supplementary Figure 1A-B**. Purification of the his-tagged BITEs was performed in a two-step process using immobilized nickel chelate chromatography (Ni Sepharose High Performance, Cytiva, GE Healthcare Life Sciences) followed by size exclusion chromatography on an Äkta Purifier with a Superdex Increase 10/300 GL column (Cytiva, GE Healthcare Life Sciences).

Cell lines and cell culture

The human AML cell lines KG1a (ATCC-CCL-246.1) and Kasumi-1 (ATCC-CRL-2724), and ALL cell line NALM-6 (ATCC-CRL-3273) were cultured in RPMI-1640 medium (Thermo Fisher

Scientific) supplemented with 10% fetal bovine serum (FBS; HyClone) and 1% penicillin/streptomycin (Gibco, Life Technologies), hereafter referred to as complete RPMI medium. The mouse fibroblast cell line NIH/3T3 (ATCC- CRL-1658) was cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific) supplemented with 10% FBS (HyClone). hCMEC/D3 cells (Nordic Biosite; 177-CLU512) were cultured to confluence in rat-tail collagen type I (Sigma-Aldrich; C3867-1VL) coated tissue culture flasks in Lonza Walkersville EGM-2-MV BULLETKIT medium (Fisher Scientific; CC3202), supplemented with HEPES (10mM), basic fibroblast growth factor (bFGF; 200ng/ml), hydrocortisone (1.4 uM), ascorbic acid (5ug/ml), penicillin-streptomycin (1%) and FBS (5%). All cell lines were cultured according to standardized cell culture procedures.

Binding assay

Cell lines (KG1a, Kasumi-1, NALM-6 or NIH3T3) or purified T-cells were incubated with CD34-specific BiTE at 1 μg/ml for 20 minutes at 4°C. Unbound antibodies were washed and the secondary anti-His tag antibody Alexa Fluor-647 (clone 4E3D10H2/E3, Thermo Fisher Scientific) was added and incubated for 15 minutes at 4°C, in addition to anti-CD34 APC (581, BD Biosciences) and anti-CD3 PE-Cy7 (SK7, BD). Cells were washed and acquired by the CytoFLEX cytometer (Beckman Coulter).

T-cell isolation and *in vitro* killing assay

PBMCs were isolated from buffy coats or donor lymphocyte infusions (DLI) via density gradient centrifugation (Lymphoprep) and banked in liquid nitrogen in compete RPMI medium with 10% DMSO. After thawing, PBMCs were recovered overnight in complete RPMI medium. T-cells were isolated via negative selection through magnetic cell sorting (Pan T-Cell

Isolation Kit; Miltenyi Biotec) and used immediately in the assays. T-cell cytotoxicity was assessed by FACS using purified T-cells from PBMCs as effector cells and cancer cell lines (KG1a, Kasumi 1, NALM-6), primary AML blasts or hCMEC/D3 as target cells. Prior to co-culture, target cells were stained with 2μM of CellTrace violet (ThermoFisher) according to the manufacturer's instructions and used immediately in the assays. Cells were co-cultured with an Effector:Target ratio of 3:1 for 48h (cancer cell lines and hCMEC/D3) and 72h (primary AML samples), and serial dilutions of αCD34, CD34/CD3 and RSV/CD3 BITEs. After incubation, cells were stained with anti-CD34 FITC (clone 581), anti-CD3 PE-Cy7 (SK7), anti-CD4 Alexa Fluor 700 (RPA-T4), anti-CD8 V500 (RPA-T8), anti-CD25 PE (M-A251) and anti-CD69 APC-Cy7 (FN50) antibodies (all from BD). Cells were washed and incubated with anti-Annexin V APC (BD) and 7AAD (BD) in Binding buffer 1X (BD) for 15 minutes at room temperature. Target cell killing was assessed by gating on CellTrace+ fraction and evaluating the Annexin V and 7AAD staining. Specific cytotoxicity (%) was calculated as: 100−(viable cells of treatment group × 100/viable cells of untreated control group).[1,2]

Single cell killing assay

PBMCs were isolated from buffy coats from healthy donors (n=6) via density gradient centrifugation with Ficoll and banked in liquid nitrogen in heat inactivated FBS with 10% DMSO. After thawing, PBMCs were recovered overnight in complete RPMI medium. T-cells were isolated and cultured in RPMI-1640 medium (Thermo Fisher Scientific) with 10% heat-inactivated FBS (Sigma-Aldrich), 1% HEPES (Sigma-Aldrich), IL-2 (60 IU/mL) and IL-15 (10ng/mL) (PeproTech and R&D Systems, respectively) for 7 days. Time-lapse live single-cell imaging was performed similarly as previously described.[3,4] In short, T-cells were stained with 5μM CellTrace yellow and KG1a target cells with 0.5 μM CellTrace far red (both from

ThermoFisher Scientific) at 37°C for 20 minutes. Stained T-cells and target cells were seeded onto a silicon-glass microchip containing four separate compartments, each with 2288 microwells with the dimensions 80 x 80 x 300 μm^3 (**Supplementary Figure 3**). The compartments contained either CD34/CD3 (1000 ng/mL) or RSV/CD3 BiTE (1000 ng/mL) or were left untreated. Imaging of the microchip was performed at 10x magnification using a confocal (Zeiss LSM 880) or widefield (Zeiss Axio Observer 7) microscope, both equipped with environmental chambers kept at 37°C and 5% CO2. T-cell dynamics and target cell killing were monitored for 21 hours, with a time resolution of 5 minutes. Briefly, T-cell-mediated target killing was detected either manually by assessing membrane integrity (e.g., blebbing) or by SYTOX Green Nucleic Acid Stain (25mM, ThermoFisher) fluorescent signal from dead cell in the wells and used to quantify the percentage of cytotoxic T-cells over time. The tracking of individual T-cells dynamic was performed manually in wells with Effector:Target ratio of 1:1 to collect information about time to first contact, contact duration, number of contacts and the percentage of cytotoxic T-cells and of lytic contacts. The percentage of cytotoxic T-cells was assessed after correction for the percentage of spontaneous death in each condition (wells containing only a single target cell that died during the assay).

Human primary AML samples

The blood samples from 14 AML patients were collected at Princess Margaret Cancer Centre (Toronto, Canada). PBMCs were cryopreserved and $CD34^+$ AML blasts used as target cells in killing assays. Purified T-cells from DLI were used as effector cells. A first cohort of six AML patients was selected and cells were co-cultured in an Effector:Target ratio of 3:1 for 72h in the presence of 2.5ug/ml of αCD34 or CD34/CD3 BiTE. A second group of eight AML patients was selected and cells were incubated with RSV/CD3 or CD34/CD3 BiTE at 2.5ug/ml

concentration for 72h. Bone marrow samples from three AML patients were collected at Karolinska University Hospital (Huddinge, Sweden). Cells were co-cultured with T-cells (n= 5) isolated from heathy donor buffy coats in an Effector:Target ratio of 3:1 for 72h in the presence of serial dilutions of CD34/CD3 BiTE. All patients provided informed consent in accordance with the Declaration of Helsinki and with approval of the ethical committee in the respective centers. After incubation, AML blasts cells were quantified by FACS using anti-CD34 FITC (clone 581). Anti-CD3 PE-Cy7 (SK7), anti-CD25 PE (M-A251) and anti-CD69 APC-Cy7 (FN50) antibodies (all from BD) were used to assess T-cells activation. Median fluorescence intensity (MFI) values were used to quantify CD34 expression levels. Blast reduction was calculated as: 100 x (1 - %CD34 BITE treated / %CD34 No Ab control).

Human hematopoietic stem cell depletion assay

Unmanipulated peripheral blood stem cell graft (PBSC) samples were collected during preparation of graft for hematopoietic stem cell transplantation (HSCT) and processed as previously described.[5] PBMCs were isolated and banked in liquid nitrogen as described above. Before usage, PBMC were thawed, rested overnight before HSCs were purified by positive selection of $CD34^+$ cells using the CD34 MicroBead Kit UltraPure (Miltenyi Biotec). T-cells were purified from the same graft using negative selection (Pan T-Cell Isolation Kit; Miltenyi Biotec). Immediately after purification, T-cells and HSCs were seeded at a 3:1 ratio and culture for 48h with serial dilutions of αCD34, CD34/CD3 and RSV/CD3 BITEs. Cells were then stained with anti-CD3 PE-Cy7 (clone SK7), anti-CD4 Alexa Fluor 700 (RPA-T4), anti-CD8 V500 (RPA-T8), anti-CD25 PE (M-A251), anti-CD69 APC-Cy7 (FN50) anti-CD34 FITC (581) and anti-CD45 V450 (HI30), all from BD. T-cell activation was assessed by CD25 and CD69 expressions and the depletion of $CD34^+$ HSCs by gating the $CD45^{dim}CD34^+$ population.

***In vivo* xenograft model**

CD34-specific BiTE antitumor efficacy and safety was evaluated in 6–8-week-old NOD.Cg-*Prkdc*^{scid}*il2rg*^{tm1Wjl}Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSG-SGM3) mice (Taconic). All the experiments were performed according to the Swedish Animal Welfare Ordinance and approved by the local animal ethical committee (Ethical approval ID1533). The hCD34^{+} KG1a cell line was inoculated intravenously (i.v.) into NSG-SGM3 mice (5x10^{6} cells at day 0). Three days after KG1a inoculation (day 3), 1x10^{7} T-cells were injected intraperitoneally (i.p.) followed by daily intravenous injections of 1.0 mg/kg anti-CD34 BiTE for 19 days (day 3-21). A second cycle of 1x10^{7} T-cells followed by BiTE was given day 10. Treatment with T-cells i.p. followed by PBS (saline) instead of BiTE i.v. served as a control for the BiTE. Groups of five mice each were used. The presence of KG1a and T-cells were assessed by flow cytometry of peripheral blood, bone marrow and spleen collected at the predetermined termination timepoint (day 21). Processed cells from the organs were analyzed by flow cytometry using the anti-mice antibody anti-mCD45 V450 (A20) and the anti-human antibodies anti-hCD3 PE-Cy7 (clone SK7), anti-hCD25 PE (M-A251), anti-hCD34 FITC (581), anti-hHLA class I BV786 (G46-2.6) (all from BD) and the LIVE/DEAD cell marker (Invitrogen). Human cells were discriminated from mouse cells using anti-hHLA class I and anti-mouse CD45 antibodies. Among the human cells, KG1a was determined by expression of CD34 and T cells by expression of CD3.

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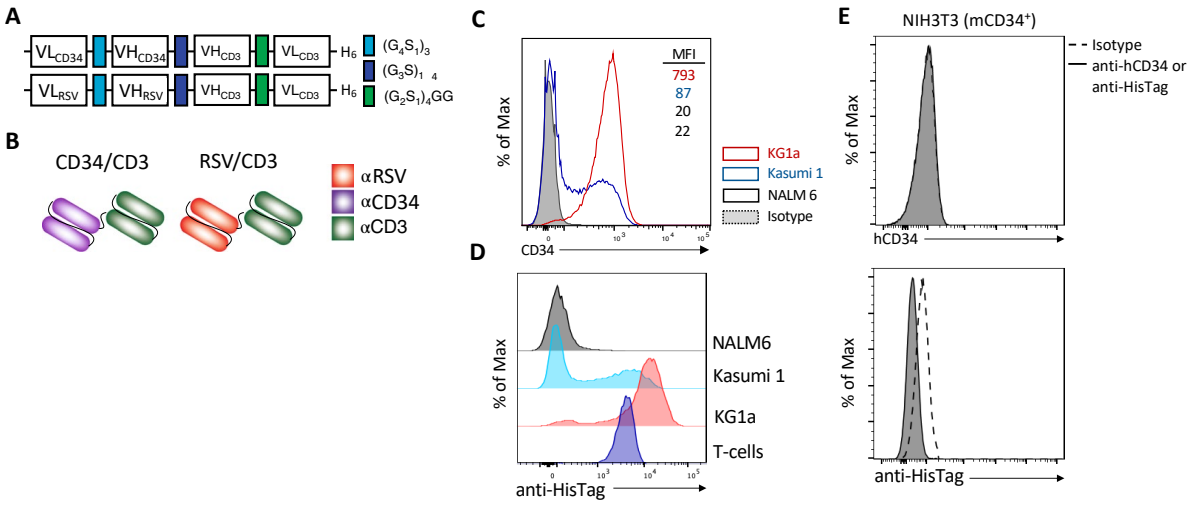
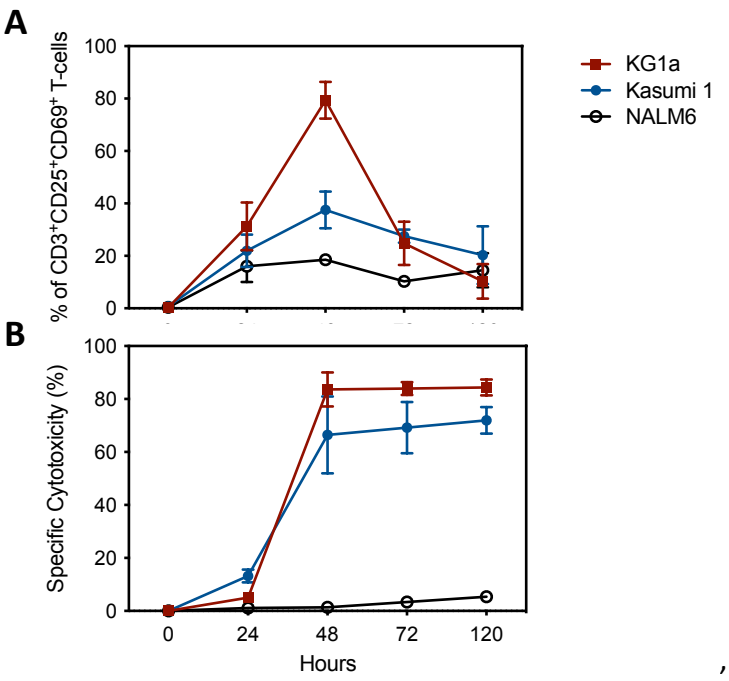
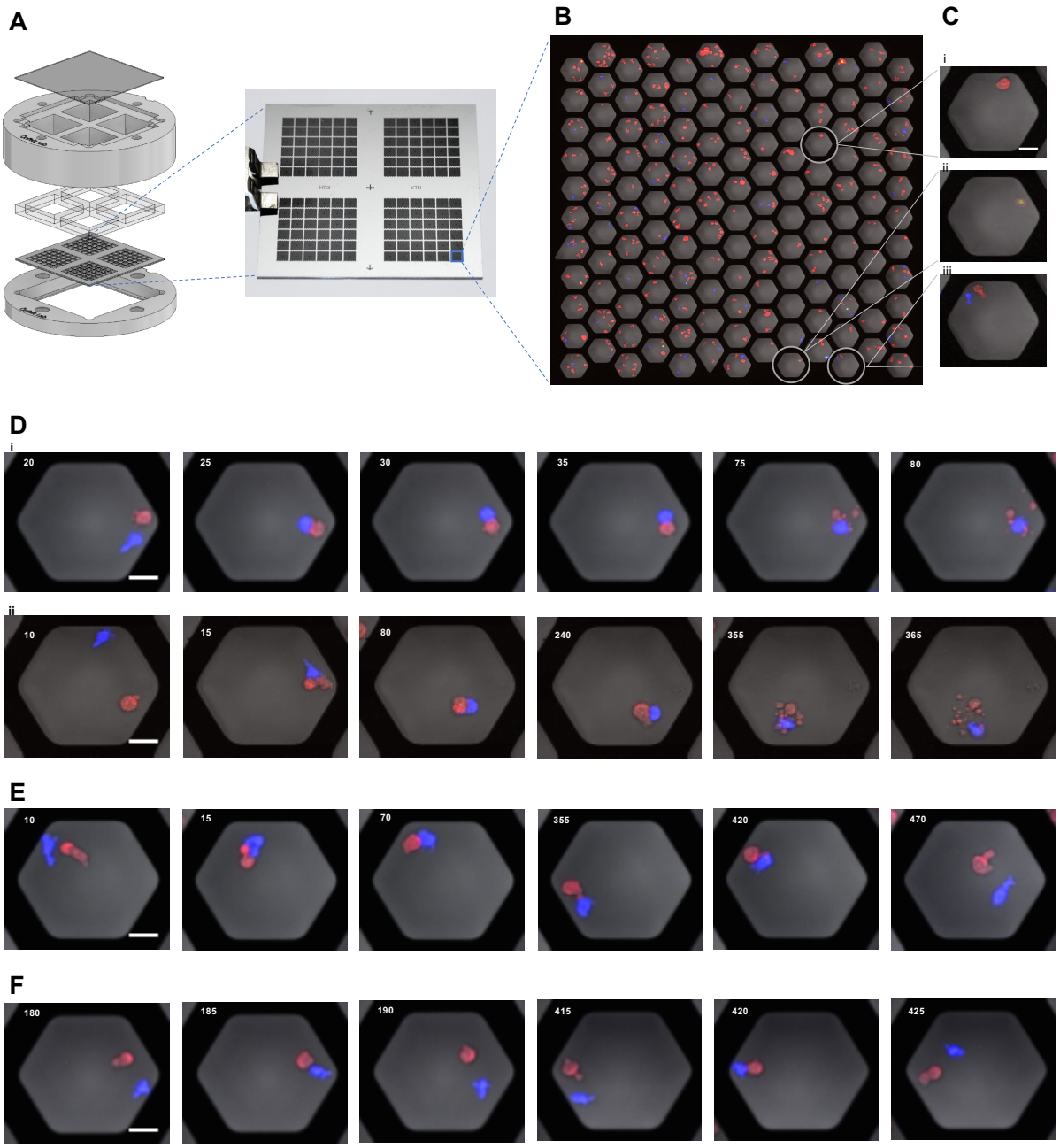


Supplementary Figure 1. BITE schematics and target binding capacity. (**A**) Schematic representation of transgenes for CD34-specific (top) and RSV-control (bottom) BITEs. The overall scFv orientation for each BITE is light-heavy-heavy-light, bridged by flexible glycine-serine linkers. A histidine tag (H_6) is present is both BITEs. (**B**) Schematic of CD34/CD3 BITE and RSV/CD3 control BITE in representative colors. (**C**) Flow cytometric histograms of CD34 expression by the cell lines KG1a (red, $CD34^+$), Kasumi 1 (blue, $CD34^{+/-}$) and NALM6 (black, $CD34^-$). Isotype control is shown in shaded gray. (**D**) Flow cytometric histograms demonstrating CD34-specific BITE binding to NALM6 (gray), Kasumi 1 (light blue), KG1a (red) and primary human T-cells (dark blue) assessed by secondary anti-histidine tag antibody detection. (**E**) Flow cytometric histograms demonstrating the absence of cross-reactivity of anti-human CD34 (upper panel) and hCD34-specific BITE (lower panel) to the mouse CD34+ cell line NIH3T3.

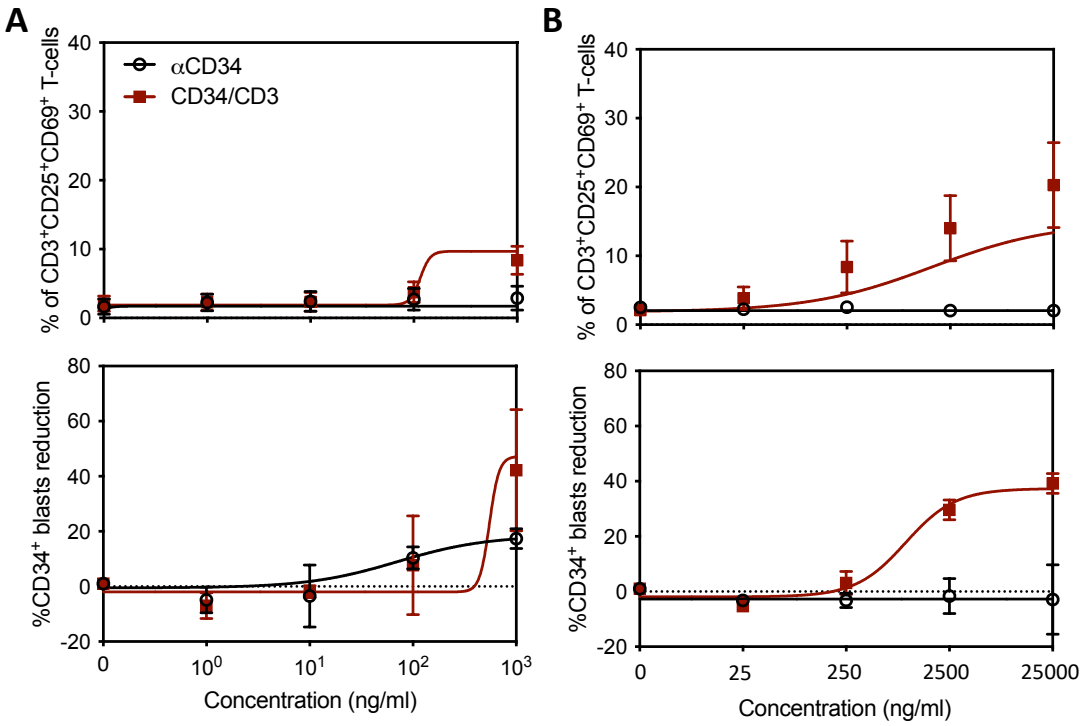


Supplementary Figure 2. T-cell activation and killing dynamics. Purified T-cells were co-cultured with KG1a, Kasumi1 or NALM6 cell lines at an E:T ratio of 3:1 in the presence of 1000ng/ml of CD34/CD3 BITE. (**A**) CD25/CD69 expressions by CD3+ T-cells and (**B**) specific cytotoxicity were assessed from 24h to 120h after co-culture by FACS (n=3, mean±SEM).

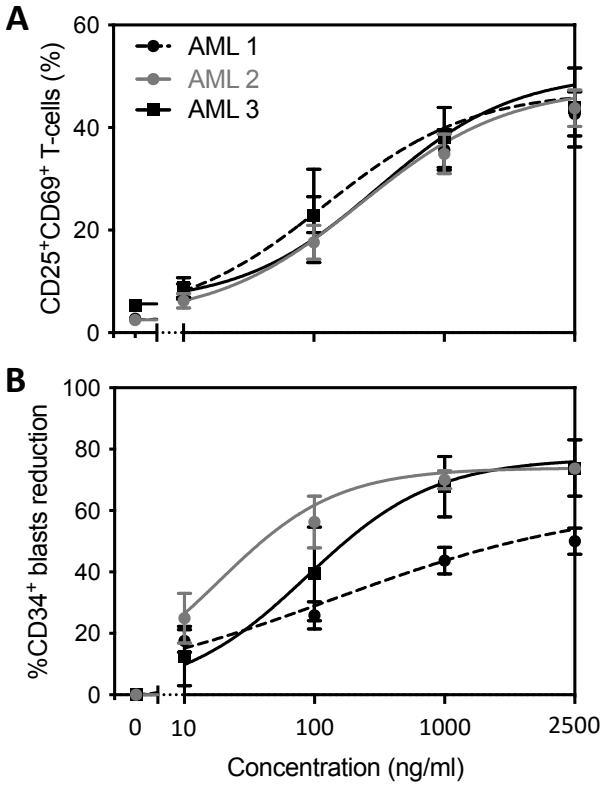


Supplementary Figure 3. Single cell killing assay. Primary T-cells were purified and stained with CellTrace yellow. KG1a target cells were stained with Far Red. The blue-stained T-cells and red-stained target cells were seeded onto a silicon-glass microchip containing four different compartments containing CD34-specific (BiTE) or RSV-specific antibodies (RSV) or left untreated (none). Images were acquired every 5 minutes over 21 hours. **A**) Illustration of microchip platform with 80 um-wide wells. **B**) A single field of view (10x objective) containing 143 individual wells. **C**) Wells with examples of: i) live target cell (stained with CellTrace far red), ii) dead target cell showing SYTOX Green staining, iii) T cell (blue, stained with CellTrace yellow) and a target cell (red). **D**) i) Example of an image sequence with BiTE (1000 ng/mL)

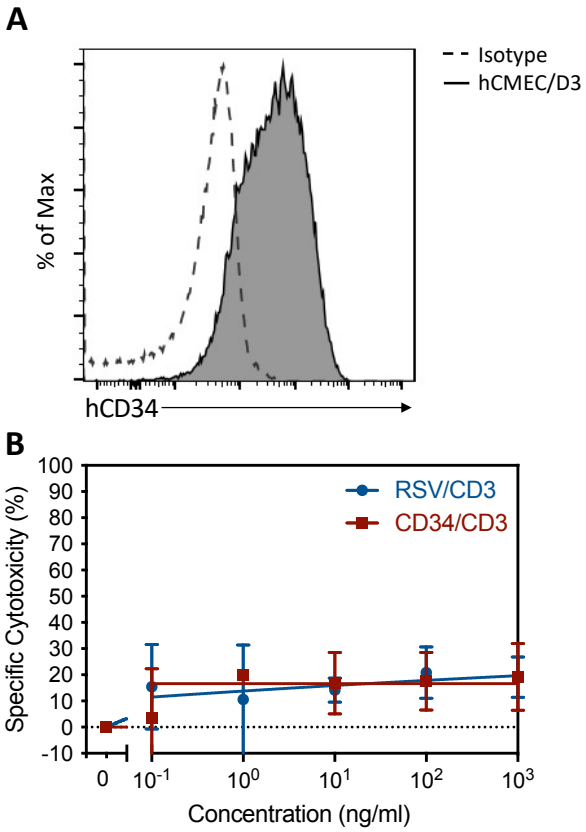
treatment where a shorter contact leads to target cell death. ii) Example of an image sequence with BiTE (1000 ng/mL) treatment where a long contact leads to target cell death. **E**) Example of an image sequence with BiTE (1000 ng/mL) treatment where a long contact does not lead to target cell death. **F**) Example of an image sequence with RSV control (1000 ng/mL) treatment where a short contact does not lead to target cell death; (in D-F, minutes indicated in white, top left corner). Scale bars = 20 μm.



Supplementary Figure 4. Primary AML cells killing dynamics and T-cell activation. Purified T-cells from DLI were co-cultured with CD34+ blasts isolated from AML patients in the presence of anti-CD34 antibody or CD34/CD3 BITE at increasing concentrations for 48h (A, n=3) or 72h (B, n=5). CD25/CD69 expressions by CD3+ T-cells (upper panels) and CD34+ blasts reduction (lower panels) were assessed by FACS (mean±SEM).



Supplementary Figure 5. CD34-specific BITE promotes BM blasts depletion. Purified T-cells from buffy coats were co-cultured with BM from three AML patients in the presence of CD34/CD3 BITE at increasing concentrations for 72h (n=5 donors). (**A**) CD25/CD69 expressions by CD3+ T-cells and (**B**) CD34+ blasts reduction (lower panels) were assessed by FACS (mean±SEM).



Supplementary Figure 6. CD34 BITE does not deplete hCMEC/D3. (**A**) CD34 expression by the human endothelial cell line hCMEC/D3. (**B**) Purified T-cells were co-cultured with hCMEC/D3 at an E:T ratio of 3:1 in the presence of RSV/CD3 (blue) or CD34/CD3 (red) BITEs. Dose response killing was assessed 48h later by FACS (n=3, mean±SEM).