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ORIGINAL RESEARCH

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Selective lysis of acute myeloid leukemia cells by CD34/CD3 bispecific antibody through the activation of γδ T-cells

Faisal Al Agrafi[a,b], Ahmed Gaballa[b,c], Paula Hahn[b], Lucas C. M. Arruda[b], Adrian C. Jaramillo[b], Maartje Witsen[b], Sören Lehmann[b,d], Björn Önfelt[e], Michael Uhlin[b,f], and Arwen Stikvoort[b]

[a]Healthy Aging Research Institute, King Abdulaziz City for Science and Technology (KACST), Riyadh, Kingdom of Saudi Arabia; [b]Department of Medicine Huddinge, Karolinska Institutet, Center for Hematology and Regenerative Medicine, Stockholm, Sweden; [c]Department of Biochemistry and Molecular Biology, National Liver Institute, Menoufia University, Shebeen El-Kom, Egypt; [d]Department of Medical Sciences, Hematology, Uppsala University Hospital, Uppsala, Sweden; [e]Department of Applied Physics, Science for Life Laboratory, KTH Royal Institute of Technology, Stockholm, Sweden; [f]Department of Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden

ABSTRACT

Despite the considerable progress in acute myeloid leukemia (AML) treatment, relapse after allogeneic hematopoietic stem cell transplantation (HSCT) is still frequent and associated with a poor prognosis. Relapse has been shown to be correlated with an incomplete eradication of CD34+ leukemic stem cells prior to HSCT. Previously, we have shown that a novel CD34-directed, bispecific T-cell engager (BTE) can efficiently redirect the T-cell effector function toward cancer cells, thus eliminating leukemic cells *in vitro* and *in vivo*. However, its impact on γδ T-cells is still unclear. In this study, we tested the efficacy of the CD34-specific BTE using *in vitro* expanded γδ T-cells as effectors. We showed that the BTEs bind to γδ T-cells and CD34+ leukemic cell lines and induce target cell killing in a dose-dependent manner. Additionally, γδ T-cell mediated killing was found to be superior to αβ T-cell mediated cytotoxicity. Furthermore, we observed that only in the presence of BTE the γδ T-cells induced primary AML blast killing *in vitro*. Importantly, our results show that γδ T-cells did not target the healthy $CD34^{intermediate}$ endothelial blood–brain barrier cell line (hCMEC/D3) nor lysed CD34+ HSCs from healthy bone marrow samples.

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Acute myeloid leukemia; bispecific antibodies; cancer immunology; CD34; γδ T-cells

Introduction

Acute myeloid leukemia (AML) is an aggressive heterogenous malignancy characterized by the accumulation of immature myeloid precursors.[1,2] Despite recent advances in the understanding of the molecular basis underlying AML pathogenesis, the current standard of care of therapy for AML patients still relies mainly on the use of conventional and highly toxic chemotherapeutic drugs.[3] The 5-y survival of AML remains poor and only a third of patients achieve complete remission.[4] Additionally, there is no effective standard treatment for relapse in the majority of AML patients, resulting in poor prognosis with little improvement in the past decades.[5,6] One of the main reasons behind the high relapse rate is thought to be caused by various chemotherapy resistance mechanisms of leukemic stem cells (LSCs),[7–10] A high LSC burden has been associated with an increased risk of relapse,[11,12] and as such, LSCs can be considered an important target for future therapies.

Bispecific T-cell engagers (BTEs) represent a novel class of therapeutic agents for solid and hematological cancers.[13,14] BTEs generate an artificial cytolytic synapse which leads to the specific lysis of target-positive tumor cells by effector T-cells via a dual-binding interaction[15–17] While previous studies have shown that LSCs are resistant to most conventional NK and T-cell mediated therapies, mainly due to a low expression of surface receptors,[18,19] BTEs could potentially bypass this issue by forcing an interaction between effector and target cell. The specificity of the BTE should, therefore, result in a more targeted and less overall toxic treatment.

CD34 is highly expressed by healthy hematopoietic stem cells (HSCs), LSCs, AML blasts, and, to an intermediate degree, by some endothelial cell populations.[20] Moreover, an increase in the expression of CD34 on AML blasts has been shown to correlate to multidrug resistance and high relapse rates.[21,22] CD34 is therefore an interesting target in AML therapies. By specifically targeting $CD34^{+}$ cells, BTEs could be used to treat AML as an adjuvant treatment before allogeneic HSCT or to make high-risk patients eligible for a second transplantation.

In our pre-clinical investigation of a novel CD34-specific BTE, we demonstrated its ability to selectively lyse AML cell lines and leukemic blasts *in vitro* and reduce tumor burden *in vivo*.[23] Moreover, CD34 intermediately positive endothelial cell lines were not killed, suggesting its non-toxicity toward these cells. In our previous investigation, BTEs were tested with conventional T-cells. As chimeric antigen receptor (CAR) therapy has shown, systemic T-cell activation may

CONTACT Arwen Stikvoort Arwen.Stikvoort@ki.se Department of Medicine Huddinge, Karolinska Institutet, Center for Hematology and Regenerative Medicine, ANA Futura, Alfred Nobel Allé 8, plan 7, Huddinge, Stockholm 141 52, Sweden
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have potential unwanted side effects[24–26] However, γδ T-cells, an unconventional subset of T-cells, are generally seen to be safe and well tolerated in several solid cancer trials.[27,28] Increased γδ T-cell levels have also been associated with favorable clinical outcomes post-HSCT,[29,30] and have demonstrated a significant cytotoxic capacity against cancer cell lines *in vitro*.[27] Although γδ T-cells only constitute a small fraction of peripheral blood (~5%), they can be found in much higher frequencies in peripheral tissues and have a pivotal role in dendritic cell and CD8 T-cell cell activation via cytokine production.[31–33] Moreover, γδ T-cells do not require MHC activation for antigen presentation and can recognize and lyse tumor cells in a non-MHC-restricted fashion.[31–33]

In this study, we assessed the biological activity of CD34/CD3 BTE on γδ T-cells *in vitro*. We demonstrated that the CD34-specific BTE selectively binds to γδ T-cells and mediates cytotoxicity of γδ T-cells against $CD34^+$ cell lines and AML blasts. Our results suggest that this novel BTE may be effective as an adjuvant therapeutic agent to treat AML patients pre-HSCT.

Materials and methods

BTE and control antibody constructs

CD34/CD3 and RSV/CD3 BTEs were generated and purified as described in detail previously.[23]

Cell lines and cell culture

The $CD34^+$ human cancer cell lines SUPB15, Kasumi, and KG1a, and the $CD34^-$ cell-line NALM-6 were obtained from ATCC. Cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific) and supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone) and 1% penicillin/streptomycin (P/S; Gibco, Life Technologies) (complete culture medium) at 37 °C in 5% CO_2 in all experiments. 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 (10 mM), basic fibroblast growth factor (bFGF; 200 ng/ml), hydrocortisone (1.4 uM), ascorbic acid (5ug/ml), penicillin–streptomycin (1%), and FBS (5%).

Sample collection

Buffy coats ($n = 22$) were obtained from healthy volunteers from Karolinska University Hospital, Sweden. Additionally, peripheral blood samples from three AML patients and bone marrow (BM) samples from three healthy donors were collected. Mononuclear cells (MCs) were isolated by Ficoll-paque-based (GE Healthcare) density gradient centrifugation following manufacturer's instructions and stored at −192° C in complete culture medium with 10% dimethyl sulfoxide (DMSO, Sigma) until further use. Informed consent was received from all subjects in accordance with the Helsinki Declaration, and the study was approved by the Regional Ethical Review Board of Stockholm, Sweden (2010/1496–31/3).

γδ T-cell in vitro expansion

MCs were thawed and cultured in complete culture medium supplemented with recombinant human IL-2 (300 IU/ml; Miltenyi Biotec) for 12 d. Zoledronic acid (5 μM, Sigma) was used on day 0 to activate γδ T-cells. IL-2 and complete culture medium were replenished twice a week and cells were transferred into new flasks, depending on cell growth. During and after expansion, cells were assessed for γδ T-cell purity and phenotypic changes by flow cytometry. Further purification was performed for samples with less than 80% of expanded γδ T-cells using negative selection by magnetic beads of γδ T-cells, following the manufacturer's instructions (TCRγ/δ+ T-Cell Isolation Kit; Miltenyi Biotec). γδ T-cells were frozen at −192 °C as described.

αβ T-cell collection

MCs were thawed and cultured in either just complete culture medium or supplemented with OKT3 (500 ng/mL, 2×10^6 cells/mL; BioLegend) overnight. T-cell purification was performed the next day using negative selection by magnetic beads, following the manufacturer's instructions (Pan T-Cell Isolation Kit; Miltenyi Biotec). Cells were assessed for αβ T-cell purity by flow cytometry and used fresh in further experiments. A minimum of 90% αβ T-cell purity was required to be included in the experiments.

Cytotoxicity assay

Target cell killing by effector T-cells was performed as described previously.[23] In short, effector and target cells (cancer cell lines, primary AML, or healthy BM) were thawed, and allowed to rest overnight at 37° C in complete culture medium. The following day, effector γδ T-cells, αβ T-cells, and αβ T-cells pre-stimulated with OKT3, were co-cultured with target cells at a 3:1 ratio with serial dilutions of BTE (0.1, 1, 10, 100, and 1000 ng/mL) for 4 h, 24 h, 48 h, 72 h, and 120 h. Target cells (4 h, 24 h, 48 h and 72 h assays) or effector cells (120 h assay) were pre-stained with 2 uM of CellTrace Violet (Thermo Fisher), according to manufacturer's instructions, to help differentiate between target and effector cells by flow cytometry. Specific cytotoxicity was calculated by dividing the percentage of viable target cells in the treatment group by percentage of viable target cells in the untreated control group. After co-culture, supernatant was saved and frozen at −80° C, while cells were analyzed by flow cytometry.

Proliferation assay

The proliferation capacity of expanded effector γδ T-cells in response to exposure to target cancer cell lines and BTE was assessed as described previously.[23] In short, effector γδ T-cells were pre-stained with 2 uM of CellTrace Violet and incubated for 5 d with target cells (SUPB15, Kasumi, KG1a, and NALM-6) at a 3:1 ratio with serial dilutions of BTE

(0.1, 1, 10, 100, and 1000 ng/mL). The fraction of CellTracelow proliferating γδ T-cells was measured by flow cytometry.

Flow cytometry

Flow cytometry was used to quantify the expression of extracellular markers. Briefly, cells were incubated with titrated volumes of antibodies in PBS for 20–30 min at 4 °C, washed with PBS, and stained with 7-amino actinomycin D (7-AAD) for 10 min at room temperature. Cells were acquired on the CytoFLEX cytometer (Beckman Coulter), and data was analyzed using FlowJo V10 (BD Bioscience). An example of the gating strategy employed is shown in Figure S1. The following antibodies were used: CD34-FITC (clone 581), CD38-PECF594 (HIT2), CD45-PE-Cy7 (HI30), CD56-AlexaFluor700 (B159), CD69-BV785 (FN50), CD94-FITC (HP-3D9), CD158b-BV510 (DX27), HLA-DR-APC-Cy7 (G46–6), NKG2D-BV650 (1D11), IgG1-isotype-control-FITC (MOPC-21) and 7-AAD from BD Biosciences; CD3-BV510 (UCHT1) and CXCR3-BV785 (G025H7) from Biolegend; TCRγδ-PE (REA591), TCRαβ-FITC (BW242/412), and TCR-Vδ2-Vioblue (123R3) from Miltenyi Biotec; TCR-Vδ1-FITC (TS8.2) and His-tag-AlexaFluor647 (4E3D10H2/E3) from Thermo Fisher Scientific.

Binding assay

The binding efficacy of BTE to γδ T-cells and cancer cell lines was measured as previously described.[23] In short, cells were incubated with BTE at a concentration of 1000 ng/mL for 20 min at 4 °C. After incubation, cells were washed with PBS, and normal extracellular staining was performed with a secondary anti-His tag antibody and relevant antibodies as described in the flow cytometry section.

FluoroSpot assay

Cytokine production by effector γδ T-cells in response to exposure to target cancer cell lines (KG1a, and NALM-6) and increasing concentrations of BTE was assessed by using the FluoroSpot assay (Mabtech). The 24 h assay was performed as described previously[34] and according to manufacturer's instructions.

Multiplex assay

Supernatant from the performed cytotoxicity assays was analyzed by multiplex assay as described previously[35] and according to manufacturer's instructions (10-plex, HCD8MAG-15K, Merck Millipore).

ELISA

Supernatant from the performed cytotoxicity assays was analyzed by ELISA according to manufacturer's instructions (ELISAPro: Human IFNg, 3420-1HP-2, Mabtech).

Statistical analyses

Dose–response cytotoxicity was assessed by Friedman test with Dunn's multiple comparisons test compared to the no BTE condition (cancer cell lines, BM & AML samples). Non-linear regression least squares fit was used to plot best fit curves where applicable. LC50 was assessed by interpolating 50% cytotoxicity from the standard curve calculated by non-linear regression. Differences in cytokine production between conditions in the FluoroSpot, Luminex, and ELISA assay were calculated by Friedman test with Dunn's multiple comparisons test compared to the relevant BTE condition. ELISA results were obtained by using the asymmetric sigmoidal standard curve to interpolate values. Expression of surface markers during γδ T-cell expansion was compared between day 0 and day 12 with the Wilcoxon matched-pairs signed rank test. Differences in proliferation between conditions were assessed by Friedman test with Dunn's multiple comparisons test compared to the relevant BTE condition. GraphPad Prism 10 was used, with significance set at $p \leq .05$.

Results

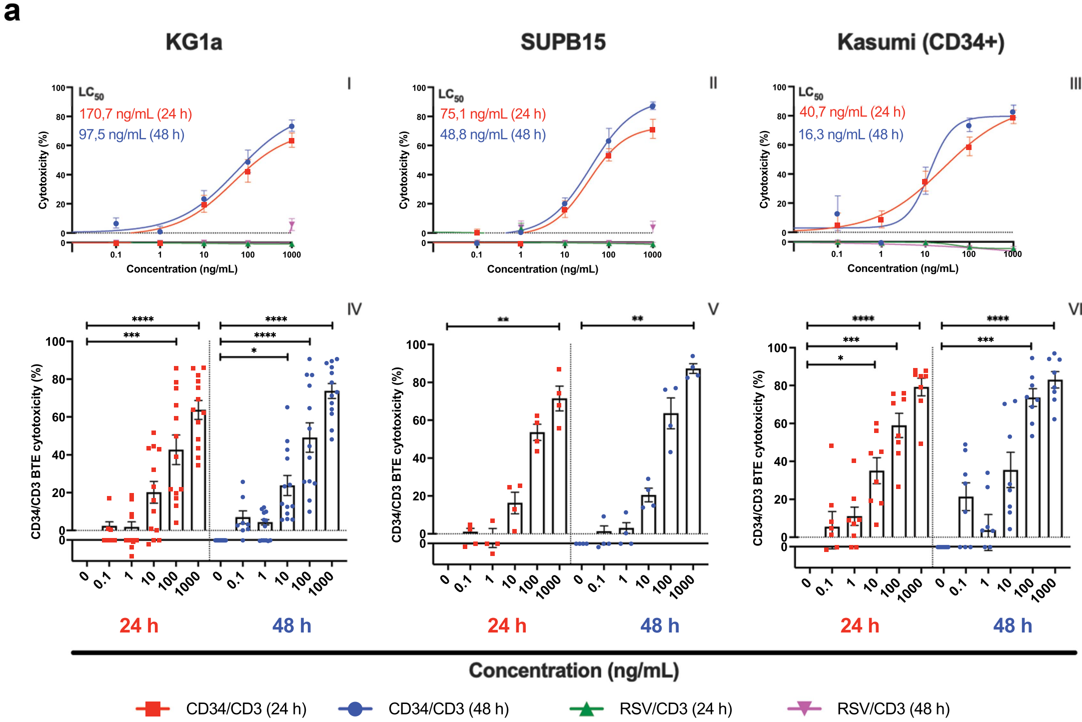
γδ T-cell expansion

During expansion, the γδ T-cell fraction increased from a median of 4% of total T-cells at day 0 to 89% at day 12 (Figure S2(a-I)). After negative selection, to further purify the γδ T-cell fraction, a median of 97% γδ T-cell purity was achieved. The Vδ2 fraction was slightly increased, with a majority of over 90% of the γδ T-cells constituting Vδ2 T-cells at day 12 (Figure S2(a-II)). A significant increase in expression of CD38, CD69, HLA-DR, NKG2D, CD56, CD158b, CD94, and CXCR3 was observed (Figure S2(b)).

CD34/CD3 BTE induces γδ T-cell-mediated lysis of CD34-expressing leukemia cell lines in a dose-dependent manner

The CD34/CD3 BTE was capable of binding to the CD34^{+} cell lines KG1a and SUPB15 without binding to the CD34^{-} cell-line NALM-6 (Figure S3(a-b)). Additionally, the CD34^{intermediate} Kasumi cell line, which expresses CD34 in two populations, was bound by the CD34/CD3 BTE in both populations (Figure S3(a-b)). Moreover, the CD34/CD3 BTE was found capable of binding to the γδ T-cells (Figure S3(b)). Meanwhile, the RSV/CD3 BTE was only able to bind γδ T-cells and not the cancer cell lines (Figure S3(b)).

To assess whether the CD34/CD3 BTE was able to mediate γδ T-cell specific killing *in vitro*, cancer cell lines were co-cultured with expanded γδ T-cells for 4 h, 24 h, 48 h, 72 h, and 120 h, in an effector-to-target ratio of 3:1 in the presence of both BTEs at increasing concentrations (Figure 1 and S4). No apparent killing was observed after 4 h of co-culture (Figure S4(a-I, a-II and a-III)). However, after 24 h and 48 h of co-culture, a dose-dependent specific killing of CD34^{+} cell lines KG1a, SUPB15, and the CD34+ population of Kasumi was observed in CD34/CD3 BTE-treated conditions (Figure 1(a)). No significant difference was observed between the 24 h and 48 h incubation periods, indicating that the γδ T-cells exert



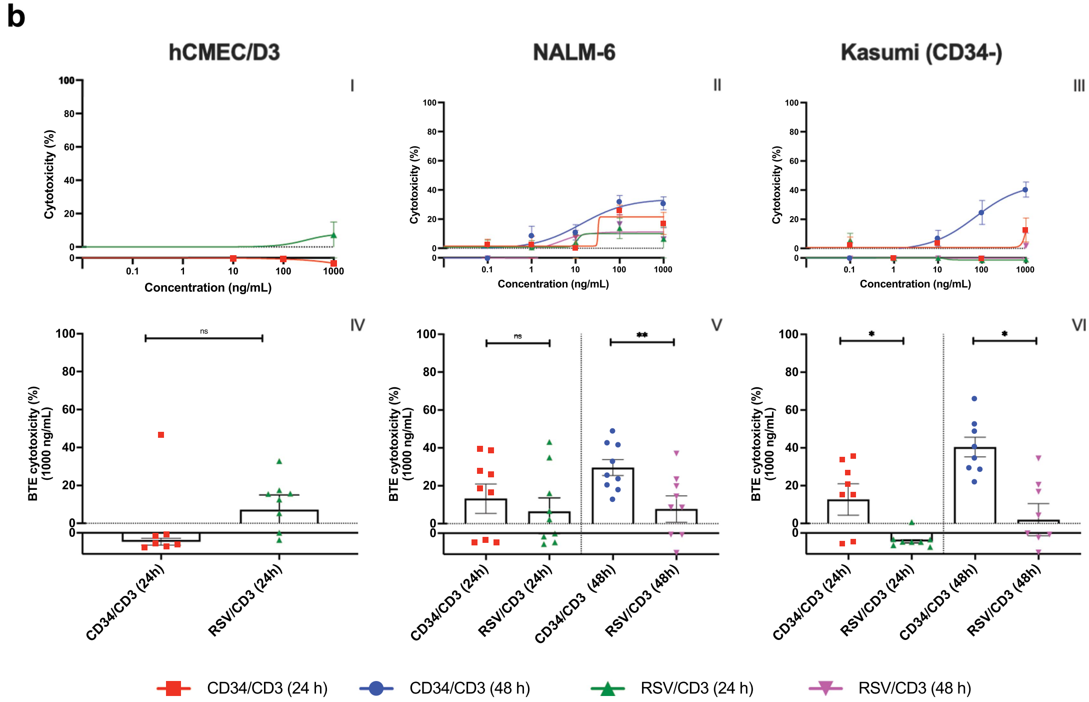


Figure 1. CD34-targeting BTE promotes CD34-specific cytotoxicity by γδ T-cells. (a) γδ T-cells were co-cultured with KG1a ($n = 13$), SUPB15 ($n = 4$) or Kasumi ($n = 8$) at an effector-to-target ratio of 3:1 in the presence of CD34/CD3 (red 24 h, blue 48 h) and RSV/CD3 (green 24 h, purple 48 h) BTE. Cytotoxicity was assessed on total target cells for KG1a and SUPB15 and on the CD34+ population of Kasumi. Dose–response killing was calculated at 24 h and 48 h by flow cytometry (mean ± SEM). (a-1, a-II and a-III) Non-linear regression least squares fit was used to plot a best fit curve. LC50 was assessed by interpolating 50% cytotoxicity from the standard curve calculated by non-linear regression. (a-IV, a-V and a-VI) Significant cytotoxicity was assessed by Friedman test with Dunn's multiple comparisons test compared to the no BTE condition. * = $p < .05$, ** = $p < .01$, *** = $p < .001$, **** = $p < .0001$. Dots represent individual donors. (b) γδ T-cells were co-cultured with hCMEC/D3 ($n = 8$), NALM-6 ($n = 9$) or Kasumi ($n = 8$) at an effector-to-target ratio of 3:1 in the presence of CD34/CD3 (red 24 h, blue 48 h) and RSV/CD3 (green 24 h, purple 48 h) BTE. Cytotoxicity was assessed on total target cells for hCMEC/D3 and NALM-6 and on the CD34- population of Kasumi. Dose–response killing was calculated at 24 h and 48 h by flow cytometry (mean ± SEM). (b-1, b-II and b-III) Non-linear regression least squares fit was used to plot a best fit curve. (b-IV, b-V and b-VI) Significant cytotoxicity was assessed by Wilcoxon (hCMEC/D3) and Friedman test with Dunn's multiple comparisons test (NALM-6 and Kasumi) comparing the CD34/CD3 BTE with the RSV/CD3 BTE at a 1000 ng/mL. ** = $p < .01$. Dots represent individual donors.

most of their cytotoxic effects between 4 h to 24 h. Additionally, no statistically significant increase in cytotoxicity was observed for the CD34^{intermediate} endothelial blood–brain barrier cell-line hCMEC/D3 at 24 h (Figure 1(b-IV)). However, an increased cytotoxicity was observed for CD34- cell line NALM-6 and the CD34- population of Kasumi when comparing the CD34/CD3 BTE to the control RSV/CD3 BTE at the highest concentration of 1000 ng/mL (Figure 1(b-V, and b-VI)). Lastly, a significant increase in cytotoxicity was observed in a dose-dependent manner of CD34^{+} cell-line SUPB15, and the CD34+ population of Kasumi after 120 h of co-culture (Figure S4(a-VI and a-VII)).

CD34-targeting BTE results in higher cytotoxicity by γδ T-cells than αβ T-cells

To assess whether the CD34/CD3 BTEs were able to mediate differences in cytotoxicity depending on whether γδ T-cells or αβ T-cells were used as the main effector cell type, cancer cell lines (KG1a and NALM-6) were co-cultured with either expanded γδ T-cells, resting αβ T-cells or αβ T-cells pre-stimulated with OKT3 for 24 h, in an effector-to-target ratio of 3:1 in the presence of both BTEs at increasing concentrations (Figure S5). A cutoff point of purity of at least 90% was set for all effector cell types and all were matched for the same donors. A dose-dependent specific killing of CD34^{+} cell-line KG1a was observed in CD34/CD3 BTE-treated conditions with a significantly increased cytotoxicity by γδ T-cells as compared to αβ T-cells (Figure S5(a and c)). Interestingly, NALM-6 cells were killed to a higher degree by γδ T-cells with the CD34/CD3 BTE, whereas αβ T-cells killed these cells particularly with the control RSV/CD3 BTE (Figure S5(d)).

CD34/CD3 BTE induces a CD34 dependent cytokine release

Next, we assessed the impact of BTE on γδ T-cell cytokine production (Figure 2, S6, S7, and S8). We used a FluoroSpot assay (Figure 2, 24 h) to detect the simultaneous release of three cytokines (IFNγ, TNFα, Granzymeβ) at the single-cell level. γδ T-cells displayed enhanced cytokine production when co-cultured with KG1a cells in the presence of high concentrations of CD34/CD3 BTE (100 and 1000 ng/ml), while limited cytokine production was observed when targeting the NALM-6 cell line or using the RSV/CD3 BTE (Figure 2(a)). Moreover, supernatant saved from the 24 h and 48 h cytotoxicity assays, was analyzed by Luminex and ELISA assay (Figure S6 and S7). The Luminex assay showed an increased production of IFNγ, TNFα, MIP1β and sFASL in the presence of high concentration of CD34/CD3 BTE (Figure S6). However, in the presence of the control RSV/CD3 BTE with KG1a cells, and both BTEs with NALM-6 cells, cytokine production increased as well, as compared to the no BTE condition (Figure S6, 24 h and 48 h). Additionally, the ELISA (Figure S7, 24 h) demonstrated an amplified production of IFNγ by the γδ T-cells with higher BTE concentrations versus the no BTE condition. Interestingly, αβ T-cells, which were not pre-stimulated, yielded the highest IFNγ production with the RSV/CD3 BTE, whereas αβ T-cells, which were pre-stimulated with OKT3, produced IFNγ to a high degree for all BTE concentrations, even without BTE (Figure S7). Lastly, Figure S8 shows that all three methods of analyzing cytokines demonstrated a similar pattern of IFNγ production by γδ T-cells with increasing concentrations of BTE.

BTE-induced interaction may induce γδ T-cell proliferation

Co-culture of γδ T cells with any cancer cell line with high concentrations of BTE, regardless of whether CD34/CD3 or RSV/CD3 BTE was used, led to an overall significant increase in γδ T-cell proliferation (Figure S9(a)). This increase in proliferation was not found to be different between target cell lines at the same BTE concentration and is thus unlikely to be linked to the lysis of the target cells (Figure S9(b)). Moreover, no difference in activated (HLA-DR+ CD69+) γδ T-cells fractions was observed after 5 d of co-culture, regardless of the target cell line or BTE used (Figure S9(c)). However, γδ T-cells already displayed a highly activated (HLA-DR+ CD69+) phenotype post 12-d expansion, with a significant increase at d10 as compared to d0 (Figure S9(c)).

BTE induces targeted killing of primary leukemic cells by γδ T-cells

We addressed whether CD34/CD3 BTE can mediate targeted killing of HSCs and AML blasts. For this purpose, γδ T-cells were co-cultured with BM-derived HSCs from three healthy donors. After 48 h, cells were harvested and the percentage of CD45^{dim} CD34^{+} HSCs was measured by flow cytometry (Figure 3(a)). Though there appears to be a small increase in cytotoxicity in BM1 (Figure 3(b-I)) for the highest CD34/CD3 BTE concentration, this increase was not found to be statistically significant. The other two BM samples did not demonstrate an increased cytotoxicity of the healthy HSCs with CD34/CD3 BTE (Figure 3(b-II and b-III)). Lastly, γδ T-cells were co-cultured with primary leukemia cells from three different AML patients. After 72 h, cells were harvested, and the percentage of CD34^{+} blasts was assessed by flow cytometry (Figure 4(a)). A dose-dependent specific killing of AML blasts in the CD34/CD3 BTE-treated conditions could be observed, while no specific killing was observed in the RSV/CD3 BTE condition (Figure 4(b-c)).

Discussion

In AML, HSCT is often used as an alternative treatment option and in some cases, it is the only curative choice. However, leukemic patients need to achieve remission before becoming eligible for HSCT. To achieve remission, high-dose chemotherapeutic drugs are given, which can have severe and deleterious short- and long-term side effects.[36,37] Furthermore, subsets of chemotherapy-resistant LSCs can survive and persist, despite high-dose pre-HSCT conditioning chemotherapy, which may lead to relapse and treatment failure.[38]

Recently, several BTEs targeting leukemias using a variety of target surface markers have been under development, with very promising *in vitro* and *in vivo* results.[39–45] We have developed a CD34-specific BTE with the aim of eradicating CD34+ HSCs and AML blasts (including LSCs) prior to HSCT.[23] The CD34-

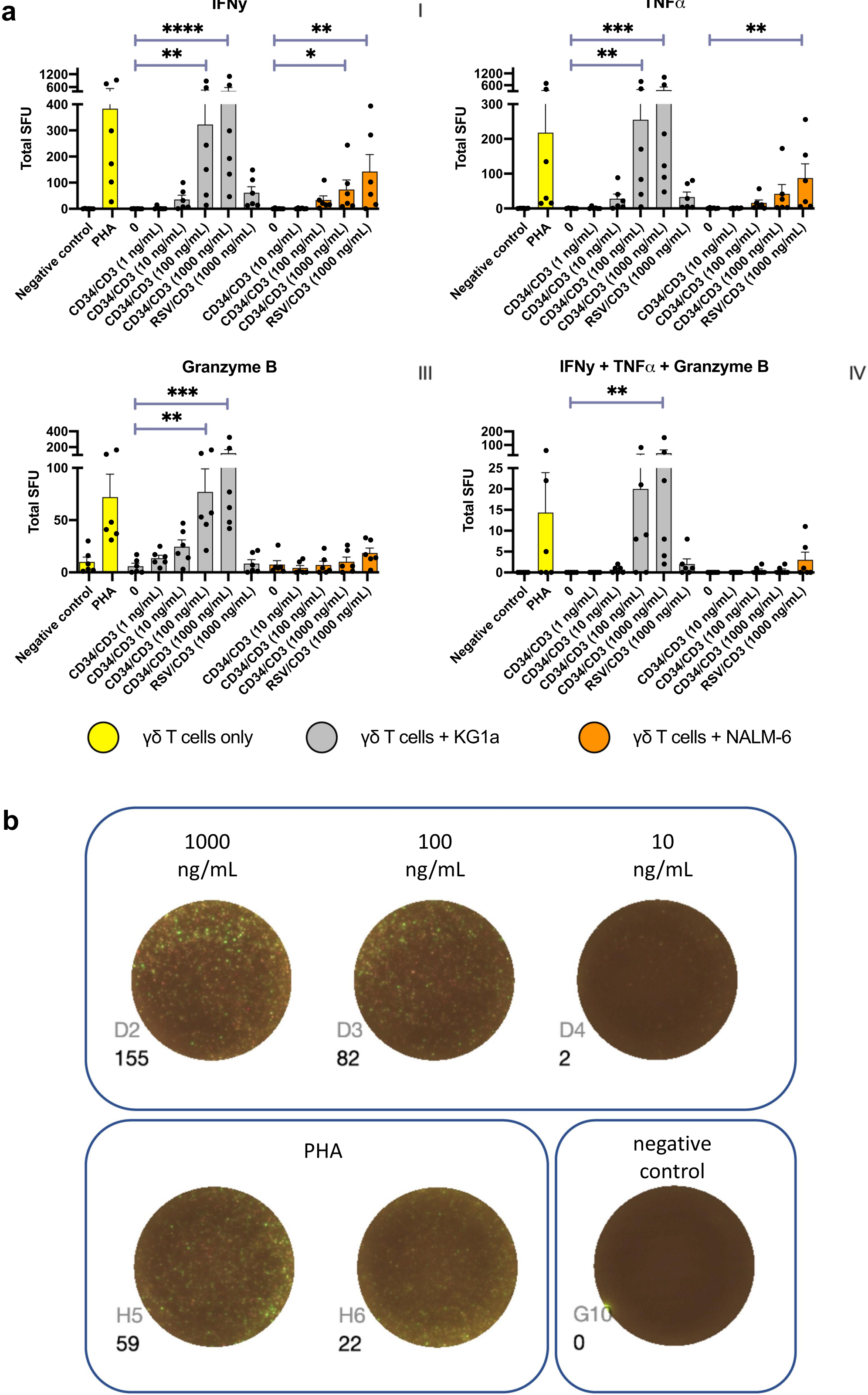


Figure 2. Cytokine analysis. (a) γδ T-cells were co-cultured with KG1a ($n = 6$) and NALM-6 ($n = 6$) at an effector-to-target ratio of 3:1 in the presence of CD34/CD3 and RSV/CD3 BTEs in a FluoroSpot plate for 24 h. Numbers of spot forming units (SFU) were measured and displayed. Significance between conditions was assessed by Friedman test with Dunn's multiple comparisons test compared to the no BTE condition. * = p <. 05, ** = p < .01, *** = p < .001, **** = p < .0001. Dots represent individual donors. (b) Representative images of FluoroSpot wells for the IFNγ + TNFα + GranzymeB with KG1a condition at three CD34/CD3 BTE concentrations (1000, 100 and 10 ng/mL).

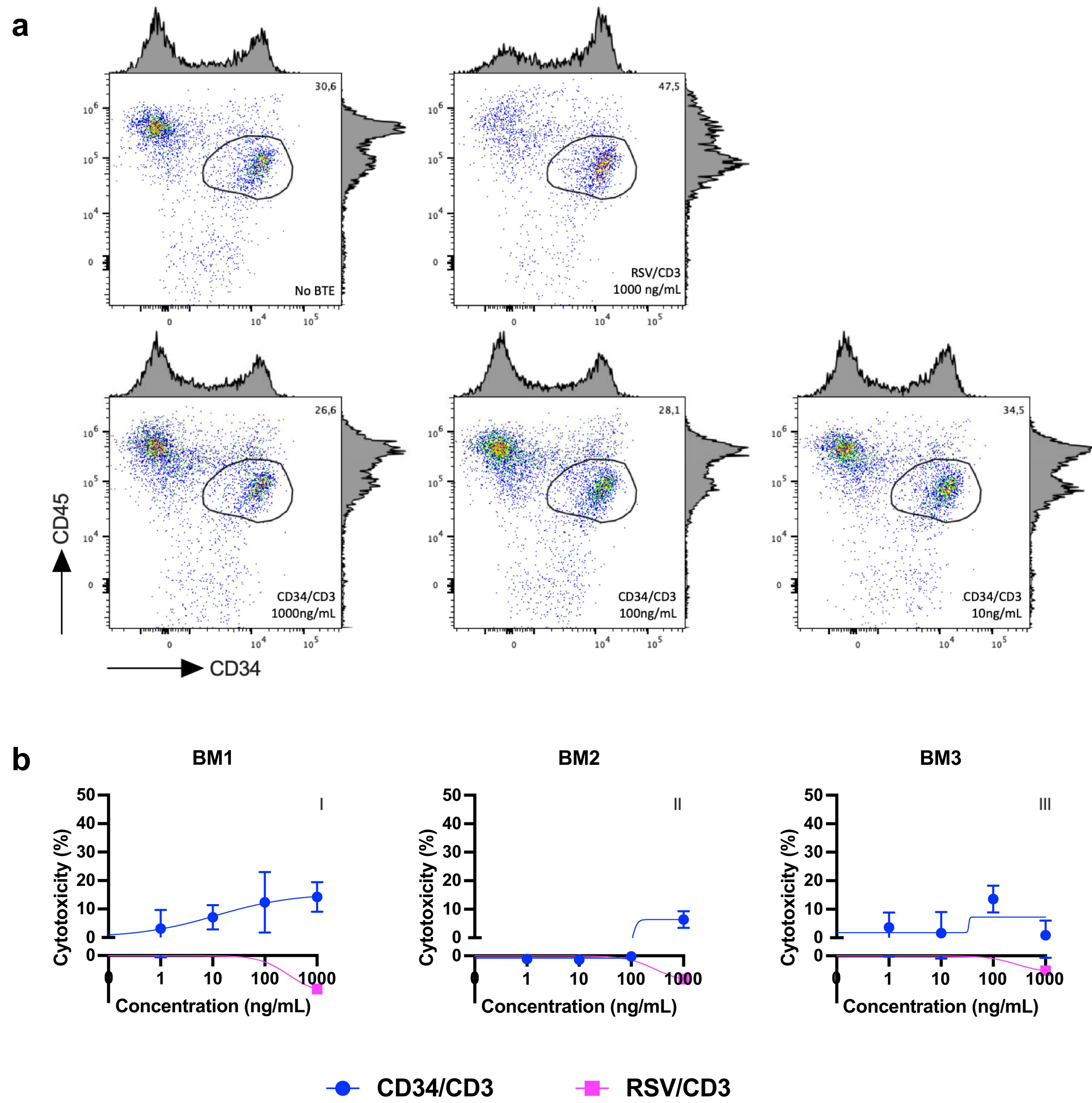


Figure 3. BTE treatment does not lead to depletion of healthy HSCs. γδ T-cells were co-cultured with BM-derived HSCs isolated from three healthy donors in the presence of CD34/CD3 BTE or RSV/CD3 at serial dilutions for 48 h ($n = 4$ γδ T-cell donors per BM sample). (a) Representative flow cytometric graphs are shown depicting the reduction in HSCs at increasing CD34/CD3 BTE concentrations. (b) Dose–response killing was calculated at 48 h by flow cytometry (mean ± SEM). Significant cytotoxicity was assessed by Friedman test with Dunn's multiple comparisons test compared to the no BTE condition.

specific BTE could, theoretically, be used in combination with chemotherapy as an adjuvant therapy before transplantation to lower LSC burden and potentially reduce the risk of an LSC-driven relapse post-HSCT. Moreover, the CD34-specific BTE may also allow for a lower dosage of chemotherapeutics to be used before transplantation in hopes of minimizing drug-related toxicities. As a result, this approach could increase the number of patients eligible for HSCT. Additionally, the long-term side effects of high doses of chemotherapeutic drugs can be severe and affect multiple organ systems.[36,37] Lowering the chemotherapeutic drug dose when used in combination with a BTE would therefore also be beneficial for children with incurable malignant hematological diseases, for which HSCT is currently the only treatment modality available.[46–48]

We have recently shown the efficacy, safety, and tolerability of a novel CD34-specific BTE *in-vitro* and *in-vivo* on depleting CD34+ AML blasts with conventional T-cells.[23] However, systemic T-cell activation may hold inherent dangers as seen in

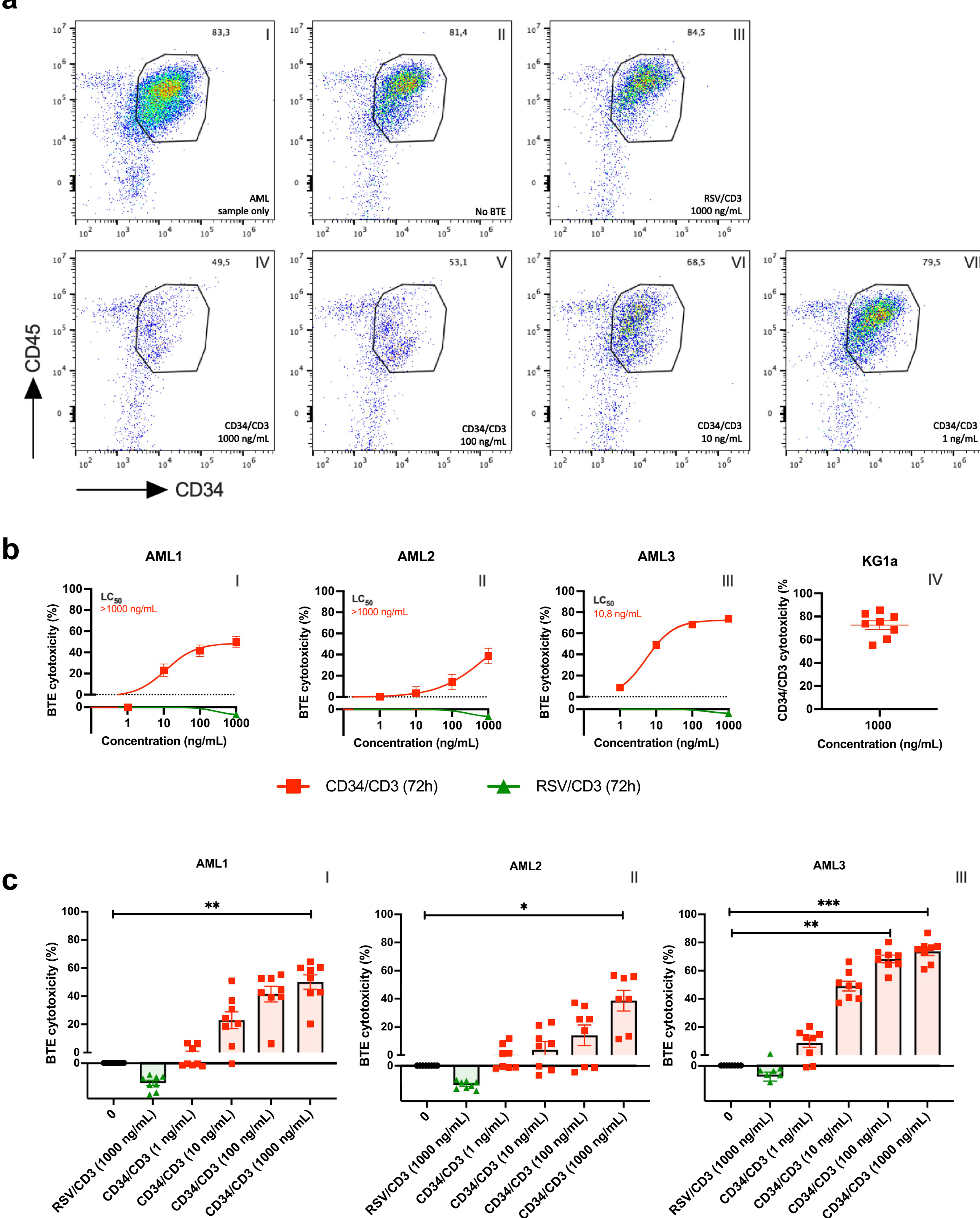


Figure 4. BTE treatment leads to depletion of patients' CD34$^+$ AML blasts. γδ T-cells were co-cultured with PBMCs isolated from three patients with AML in the presence of CD34/CD3 or RSV/CD3 BTE serial dilutions for 72 h ($n = 8$ γδ T-cell donors per AML patient). (a) Representative flow cytometric graphs are shown depicting the reduction in AML blasts with increasing CD34-specific BTE concentrations. (b-I, b-II and b-III) Dose–response killing was calculated by flow cytometry (mean ± SEM). Non-linear regression least squares fit was used to plot a best fit curve. LC50 was assessed by interpolating 50% cytotoxicity from the standard curve calculated by non-linear regression. (b-IV) KG1a was included in the assay as a positive control. Dots represent individual donors. (c) Significant cytotoxicity was assessed by Friedman test with Dunn's multiple comparisons test compared to the no BTE condition. Dots represent individual donors. * =p < .05, ** = p < .01, *** = p < .001.

CAR therapies, where cytokine release syndrome can cause a heightened inflammatory response, widespread organ dysfunction and increased mortality.[24,25] Additionally, long-term effects in patients that received B-cell targeted CAR T-cell infusions were common and included B-cell depletion, hypogammaglobulinemia, susceptibility to infection and cytopenia.[49] A common thread in most CAR therapies studied so far, is the use of conventional T-cells. However, some CAR studies have been done with γδ T-cells with promising results, and there are even several clinical trials ongoing (NCT04702841, NCT04107142, NCT04735471).[50–52] γδ T-cell therapy has also been shown to be safe and well tolerated in several solid cancer trials.[27,28]

Here, we sought to assess, as a proof-of-principle, the efficacy of our CD34-specific BTE with γδ T-cells, as a promising adjuvant in pre-HSCT treatment strategies. Due to pre-HSCT chemotherapy conditioning, and disease progression, T-cells in patients are often compromised. Therefore, any treatment requiring the use of functional T-cells would most likely necessitate a T-cell infusion as well. Since conventional T-cells have been linked to several serious side effects, it is potentially difficult to treat patients pre-HSCT with BTEs and an infusion of conventional T-cells. However, with the promising clinical results of the safety and well tolerability of γδ T-cell therapies, combining BTEs and an infusion of expanded autologous or allogeneic γδ T-cells might be a possible approach in the future.

Since the impact of our CD34-specific BTE on γδ T-cells was still unknown, we expanded γδ T-cells from healthy buffy coats for 12 d (Figure S2). During expansion, several surface activation markers such as CD69 and HLA-DR were upregulated on the γδ T-cells, which indicate a highly activated and reactive phenotype. While overall γδ T-cell frequency increased from a median of 4% to over 90% during the expansion, the Vδ1 and Vδ2 fractions remained similar, with the Vδ2 constituting the majority of the γδ T-cells. These results reflect the physiological setting, where Vδ2 T-cells constitute most of the γδ T-cells in peripheral blood.[53]

Despite extensive proliferation of γδ T-cells during the 12-d expansion, our proliferation assays revealed a further capacity of γδ T-cells to proliferate upon exposure to a high concentration of BTE (Figure S9(a-b)). This proliferation occurred independently of BTE type or target cell line used, suggesting a driving role of just the BTE binding to the CD3 receptor on the γδ T-cells at high BTE concentrations. This contrasts with our previous study, where only CD34/CD3 BTE and a $CD34^+$ cell line stimulated conventional T-cell proliferation.[23] This suggest a clinical potential for *in vitro* expansion of patient-derived γδ T-cells, followed by re-infusion together with BTEs, while retaining proliferative capacity *in vivo*.

Consistent with the previous findings from Arruda et al.,[23] we showed that CD34/CD3 BTE can induce a specific cytotoxic response by γδ T-cells against CD34 expressing AML cell lines, in a dose-dependent manner (Figure 1 and S4). Although reduced, we observed cytotoxicity toward CD34- cell line NALM-6 and the CD34- population of Kasumi at the highest CD34/CD3 BTE concentration, especially so at 48 h, indicating a potential inherent sensitivity of these cell lines to activated γδ T-cells. Importantly, the blood–brain barrier endothelial cell-line hCMEC/D3 remained unaffected by CD34/CD3 BTE even at the highest concentration. Unlike conventional T-cells, γδ T-cell mediated killing occurred mainly within the first 24 h, albeit later than 4 h. Moreover, a direct comparison between γδ T-cells and αβ T-cells revealed significantly higher cytotoxicity with γδ T-cells (Figure S5). While pre-stimulation of αβ T-cells with OKT3 increased cytotoxicity compared to resting αβ T-cells, it remained lower than that of γδ T-cells. Interestingly, CD34- cell line NALM-6 exhibited a similar susceptibility to killing by αβ T-cells with RSV/CD3 BTE as by γδ T-cells with CD34/CD3 BTE. These results suggest that the NALM-6 cell line appears to be somewhat susceptible to T-cell killing regardless of BTE or T-cell type. All in all, these results indicate that the γδ T-cell response is initiated earlier when compared to conventional T-cells, reflecting an immunobiological feature of γδ T-cells.

Additionally, an increased inflammatory cytokine production profile could be observed at high BTE concentrations with KG1a and γδ T-cells in our FluoroSpot, Luminex and ELISA assays (Figure 2, S6, S7, S8). However, cytokine production increased notably with the CD34- cell line NALM-6 or αβ T-cells, suggesting a correlation with high BTE concentrations rather than cytotoxicity. Though we did not test other BTEs, we could hypothesize that this phenomenon would also occur with other BTEs targeting CD3. Since some CD3-targeting BTEs are clinically used without many side effects, we anticipate manageable toxicities *in vivo* despite increased cytokine production. Discrepancies in cytokine production among our methods were also observed, notably lower levels in the FluoroSpot assay with NALM-6 conditions compared to Luminex and ELISA. Methodological differences, such as plate setups and cell numbers, may contribute to these differences. While we used supernatant from cytotoxicity assays that were performed in round-bottom plates, the FluoroSpot assay used flat-bottom plates, and due to constraints of the method, we used fewer cells for the FluoroSpot assay, making it harder for effector and target cells to converge. Moreover, analyzing only total spot-forming units in the FluoroSpot assay may underestimate cytokine production, warranting further investigation into spot intensity for a comprehensive assessment.

Notably, we also showed that CD34/CD3 BTE can induce a specific cytotoxic response by γδ T-cells against primary AML blasts, in a dose-dependent manner (Figure 4). The primary AML samples were used unaltered in the assays. Flow cytometry determined the percentage of donor-derived γδ T-cells in the samples, with all samples containing <1% of γδ T-cells. However, a general T-cell presence, predominantly donor-derived αβ T-cells, was detected at the start of the cytotoxicity assays, potentially influencing cytotoxicity results. Conceivable concerns may also arise considering the potential impact of γδ T-cell proliferation, particularly for the 72 h AML cytotoxicity assay. While significant proliferation of γδ T-cells occurred at 120 h with high BTE concentrations (Figure S9), no statistically significant increase in proliferation of γδ T-cells at 24 h or 48 h was observed (data not shown). Unfortunately, γδ T-cell proliferation at 72 h was not assessed. While unlikely to significantly influence AML cytotoxicity assay results, the role of γδ T-cell proliferation cannot be entirely ruled out.

portantly, our results show that γδ T-cells did not target our healthy CD34^{intermediate} endothelial blood–brain barrier cell line (hCMEC/D3, Figure 1(b-I and b-IV)) nor lysed CD34+ HSCs from healthy BM samples (Figure 3), suggesting that γδ T-cells and CD34/CD3 BTE could potentially be used safely *in vivo*.

Taken together, our results indicate a potential role for γδ T-cells and CD34/CD3 BTEs in AML therapies. Yet, further studies are still required to confirm our findings within a larger sample size and in *in vivo* pre-clinical AML models.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORCID

Arwen Stikvoort http://orcid.org/0000-0002-0251-5628

Data availability statement

Data available on request from the authors.

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