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In-depth comparative characterization identifies scalable CD123-CAR-NK cells as promising strategy to combat AML

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Article

Keywords:

Posted Date: August 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10643783/v1

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Additional Declarations: Yes there is potential Competing Interest. E.U. has no COIs directly related to this manuscript. E.U. has a sponsored research project with Gilead and BMS. No competing financial interests exist for the remaining authors. SM, JKo, JKu and CZ are employees of Miltenyi Biotec.

Abstract

Acute myeloid leukemia (AML) is a hematological malignancy characterized by immense heterogeneity, not only between patients but also within malignant populations, leading to limited curative outcomes. These challenges underscore the urgent need for innovative therapies, such as chimeric antigen receptor (CAR)-T cell therapies, which show promising antileukemic activity but remain limited by toxicity and antigen escape. In contrast, CAR-Natural Killer (NK) cell therapies offer an improved safety profile, off-the-shelf availability, and high inherent cytolytic activity. Owing to the heterogeneous nature of AML, multiple target antigens must be addressed and validated. However, there have been no side-by-side comparisons of different AML antigens targeted by the same CAR construct for NK cell-based therapy. This study addressed CD33- and CD123-targeting CAR-NK cells, both in vitro and in vivo, in a fully matched experimental setting. Functional analyses revealed a significant increase in killing efficacy of both engineered CAR-NK cell products, compared to non-transduced NK cells. Additionally, in-depth transcriptomic analyses revealed the upregulation of motility and cytotoxicity pathways in CD123-CAR-NK cells compared to CD33-CAR-NK cells. Consistent with this, only CD123-targeting CAR-NK cells increased survival in a xenograft AML model and exhibited significant effects against primary AML in vitro and in vivo. Furthermore, upscaling of CD123-CAR-NK cell manufacturing using the CliniMACS Prodigy System, resulted in highly functional CAR-NK cell preparations. Together with the transcriptional and functional profiling, this work represents an encouraging proof-of-concept for the application of genetically engineered CD123-targeting CAR-NK cell preparations in advanced AML therapy.

Introduction

Acute myeloid leukemia (AML) is one of the most aggressive hematologic malignancies, with high relapse rates and limited long-term survival, particularly in patients who experience recurrence after conventional chemotherapy and allogeneic hematopoietic stem cell transplantation.[1] Immunotherapeutic approaches, specifically chimeric antigen receptor (CAR)-engineered immune cells, have gained significant interest as potential strategy for targeting AML blasts and leukemia initiating cells (LIC).[2]

Compared to T cells, natural killer (NK) cells offer distinct advantages in CAR-based therapies, including innate antitumor activity without prior sensitization and a favorable safety profile. Notably, this includes a lower incidence of cytokine release syndrome (CRS) and neurotoxicity compared to CAR-T cells.[3] Additionally, NK cells possess the potential for "off-the-shelf" availability due to their reduced GvHD risk.[4] However, the heterogeneity of AML and the lack of leukemia-specific surface antigens pose major challenges in designing effective and safe CAR-based therapies. Among the most extensively studied targets, CD123 and CD33 have emerged as key candidates for CAR-NK cell therapy against AML, each with distinct advantages and limitations.[5]

As a myeloid differentiation antigen, CD33 is highly expressed on AML blasts and myeloid progenitors but is also present on healthy hematopoietic stem and progenitor cells (HSPCs).[6] The expression by

healthy myeloid progenitors presents a significant challenge in terms of side effects due to potential myelosuppression. Therefore, other AML targets, such as CD123, also known as interleukin-3 receptor alpha chain, have been extensively studied.[7] CD123 is overexpressed in AML blasts as well as LICs, while its expression on healthy HSPCs is significantly lower than CD33.[8] Preclinical and early clinical studies have demonstrated promising anti-leukemic activity of CD123-CAR-NK cells, with reduced CRS and neurotoxicity compared to CAR-T cell therapies.[9]

This study compared CD33- and CD123-CAR-NK cell products against an aggressive, NK-resistant AML cell line engineered to express both CD33 and CD123 on the surface. Additionally, functional cytotoxicity against patient-derived primary AML samples was assessed. Although both CAR-NK cell products exhibited similar in vitro tumor cell elimination, only CD123-CAR-NK cells increased survival and showed significant effects in vivo, in both cell line- and patient-derived xenograft (PDX) models. Additionally, single-cell RNA sequencing revealed underlying gene expression patterns, highlighting that the CD123-CAR-NK cell product showed a more tissue-adaptive and cytotoxic phenotype than CD33-CAR-NK cells. Based on these convincing results, the upscaling process for CD123-CAR-NK cells was further developed and functionally evaluated using the CliniMACS Prodigy system in small- and large-scale. This process had already been successfully established for CD33-CAR-NK cells[10], facilitating GMP-compliant, large-scale production of CAR-NK cells, which is a critical and challenging component for clinical translation[11].

This study not only explores potential underlying mechanisms that may explain the increased efficacy of CD123- and CD33-CAR-NK cell preparations in treating AML but also lays the foundation for successful clinical translation of advanced cell therapy with genetically engineered AML-targeting NK cells.

Methods

Isolation, cultivation and transduction of NK cells

Manual production

Primary NK cells were isolated from buffy coats provided by the German Red Cross Blood Donation Service (Frankfurt am Main, Germany) in line with the declaration of Helsinki, utilizing the EasySep™ Human NK Cell Enrichment Kit (StemCell, Vancouver, Canada). Lentiviral transduction was performed as previously described.[12]

Small-and large-scale production for upscaling

Large-scale produced NK cells were generated as previously described[10], utilizing the CliniMACS Prodigy System. Primary NK cells were isolated from leukapheresis obtained at Miltenyi Biotec (Bergisch-Gladbach, Germany) from healthy volunteers and in line with the declaration of Helsinki. This NK cell isolation and transduction (only large-scale) method was used for data shown in Fig. 3, 6, S2 A-E, and S5.

CAR construct

Single chain variable fragments (scFvs) were derived from My9-6 (CD33)[13] and a non-commercial antibody (CD123, Miltenyi Biotec). For surface expression of the CAR, a GM-CSFRα derived signal peptide was included. Self-inactivating, third-generation lentiviral vectors with a baboon envelope (BaEV-LVs) were generated by transiently transfecting HEK293T cells using either polyethylenimine (PEI)[14] or MACSfectin (Miltenyi Biotec).

Cytotoxicity- and repetitive tumor challenge assay

The cytotoxic effect of CAR- and NT-NK cells was assessed via flow cytometry, as previously described.[12]

For the analysis during repetitive tumor challenges, an E:T ratio of 1:1 was used, and fresh target cells were added on day 1, 3 and 5 in 50% new culture medium. The number of viable cells was measured 24 hours after each addition of target cells.

Xenograft mouse model

In vivo efficiency of (CAR-) NK cells was determined in our established xenograft model, already described in detail earlier.[12] In short, female NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) or NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ (NSG-SGM3) mice (6–16 weeks old; The Jackson Laboratory Bar Harbor, ME) were intravenously injected with $0.5x10^6$ or $1x10^6$ OCI-AML2^{CD123+} (GFP/luc) and grouped on day 1 or 2 after tumor injection based on total flux (p/s), respectively. Mice were subsequently injected with $5x10^6$ (CAR-) NK cells on day 3 and 7 (Fig. 2A-D), with $10x10^6$ (CAR-) NK cells on day 3 (Fig. 6), or day 2, 8, 11 and 15 (Fig. 2E-H). Mice received daily subcutaneous injections of 25.000 IU IL-2 (Novartis, Basel, Switzerland). All experiments were approved by the Regierungspräsidium Darmstadt, Germany.

Primary AML samples for in vitro and in vivo studies

Tumor samples and patient data used in this study were provided by the university cancer center Frankfurt (UCT). Written informed consent was obtained from all patients, and the study was approved by the institutional review boards of the UCT and the ethical committee at the university hospital Frankfurt (SHN-2-2022). Frozen primary AML cells from patients #F1-#F3, used for *in vitro* analysis, were thawed and incubated for 24 hours prior to staining and co-culture for cytotoxicity assessment (see supplemental methods for details). The ethics commission of the University Hospital Frankfurt, Germany (approval no. 274/18) approved the usage of primary patient material, in accordance with the Declaration of Helsinki.

Peripheral blood samples from patient #T1, used for PDX *in vivo* analysis, were collected at diagnosis after obtaining informed consent, in accordance with the Declaration of Helsinki. Mononuclear cells were isolated by density gradient centrifugation, viably cryopreserved in freezing medium containing 10% DMSO, and stored in liquid nitrogen. Cells were freshly thawed prior to each experiment.

PDX Model

PDX models were established as previously described [15]. Female NSG mice (11–15 weeks old; The Jackson Laboratory Bar Harbor, ME) were irradiated and injected with 1 × 10^6 primary AML cells depleted of $CD3^+$ and $CD19^+$ cells after 24 h. AML cell engraftment was monitored by intrafemoral aspiration. After confirmation of engraftment, mice received two intravenous injections of 1 × 10^7 (CAR-) NK cells on days 1 and 5. Blood samples were collected on day 1, 5, and 10. Mice received daily subcutaneous injections of 25.000 IU IL-2 (Novartis, Basel, Switzerland) and were euthanized on day 10 post-treatment. BM, spleen, liver, and blood were harvested, and analyzed by flow cytometry and histology (see supplemental methods for details).

Chromium single-cell RNA-sequencing

For detailed single-cell transcriptomics data analysis see supplemental methods.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 10.5.0. It was presumed that NK cell functionality in healthy donors and *in vivo* experiments followed a normal distribution. *In vitro* data with more than three donors was analyzed with a two-way ANOVA. For *in vivo* data matched one-way- (1–3 mice) or two-way ANOVA (> 3 mice) or Log-Rank (Mantel-Cox, Survival experiment) was used. Only significant results are shown in the figures. Significance was set as follows: $p < 0.05$*, $p < 0.01$**, $p < 0.001$***, and $p < 0.0001$****.

AI disclosure

The AI academic writing tool “Paperpal” was used to check for plagiarism, spell check and grammatical assistance, as well as to refine language. All AI-generated content was reviewed and verified by the authors.

Results

CD123- and CD33-targeting CAR-NK cells demonstrate high anti-leukemic efficacy in vitro

To gain insights into the antileukemic capacity of AML-targeting CAR-NK cells, a functional comparison of CD123- and CD33-CAR-NK cells was performed. CAR constructs were transduced into cytokine-primed NK cells using baboon envelope pseudotyped (BaEV) lentiviral vectors 2 days after isolation from PBMCs (Fig. 1A). These preparations were expanded feeder-free with low-dose IL-15 and IL-2. For comparability, CAR domains were identical, except for the scFv, derived from My9-6 for CD33-CAR or a non-commercial antibody for CD123-CAR (Fig. 1B). CAR expression (Fig. 1C) and NK cell expansion (Fig. 1D) did not differ significantly from non-transduced (NT) NK cells or between constructs. NK cell phenotype, viability, and purity confirmed the expected activated $CD16^{+}/CD3^{-}$ phenotype[10,12] with no differences between CAR- and NT-NK cells (Fig. S1). To verify cytotoxic efficacy, OCI-AML2 cells that are $CD33^{high}$ with low sensitivity to NT-NK killing, were transduced with CD123 to create a double-positive line (Fig. S2F). This "OCI-AML2$CD123^{+}$" line was used for cytotoxicity assessment after 4 and 24 h of co-culture (Fig. 1E). Both CAR-NK preparations showed increased killing versus NT-NK cells at E:T ratios of 1:1 and 5:1. CD123-CAR-NK cells induced higher target lysis than CD33-CAR-NK cells after 4 and 24 h at a 5:1 ratio (Fig. 1F). To address long-term killing and exhaustion, NK cells were repeatedly challenged with OCI-AML2$CD123^{+}$ (Fig. 1G). After 5 days, both preparations exhibited efficient killing without signs of functional exhaustion (Fig. 1H). Taken together, we demonstrated increased in vitro cytotoxic efficacy of CD123-CAR-NK cells compared to CD33-CAR-NK cells.

CD123-CAR-NK cells significantly increase survival in an AML xenograft model

Based on the promising anti-leukemic activity observed in vitro, further evaluation of CAR-NK cell function was carried out in an OCI-AML2^{CD123+} xenograft model in immunocompromised NSG-SGM3 (Fig. 2A-D) or NSG (Fig. 2E-H) mice. The first experiment (Fig. 2A) was comprised of two treatments of each 4-5x10^{6} NT-NK or CAR-NK cells on days 3 and 7 after tumor cell injection. The animals were monitored using bioluminescent imaging (BLI) twice a week (Fig. 2B). The corresponding total flux values (Fig. 2C) showed significantly reduced tumor signals in both CAR groups compared to NT-NK cell-treated or untreated (UT) animals. However, total flux values were significantly lower in mice treated with CD123-CAR-NK cells than in those treated with CD33-CAR-NK cells. The same tendency was observed in the tumor load of the bone marrow, which was assessed 21 days after tumor injection in 1–3 representative mice via flow cytometry (Fig. 2D).

To elucidate a potential survival benefit, a higher starting tumor load of 1x10^{6} cells was combined with earlier and more frequent treatment with NK cells at four doses of 10x10^{6} cells (Fig. 2E). Herein, the BLI (Fig. 2F) and the corresponding total flux (Fig. 2G) indicated a significantly less pronounced effect of CD33-CAR-NK cells on engrafted OCI-AML2^{CD123+} compared to CD123-CAR-NK cells. This was also reflected in the survival probability (Fig. 2H), which was significantly increased only in the CD123-CAR-NK cell group. Additionally, tumor load and target expression were measured in the bone marrow, spleen, and leukemic chloroma, which were observed in some mice (Fig. S4), showing a reduction of CD33 as well as CD123 positive tumor cells in the bone marrow of mice treated with CD123-CAR-NK cells. Overall,

these results suggest an advantageous effect of the CD123- compared to CD33-CAR-NK-cell product, which is more pronounced in vivo than in vitro.

Single cell RNA-sequencing reveals an activated cytokine-secreting NK cell phenotype in CD123-CAR-NK cells compared to CD33-CAR-NK and non-transduced NK cells

To gain insights into differences between CD123- and CD33-CAR-NK cell signaling, single-cell RNA sequencing was performed after 4 h of co-incubation with OCI-AML2^{CD123+}. Unsupervised clustering of integrated NK populations, including CAR-positive and -negative cells, identified four states (Fig. 3A). **Cluster 1** reflected a cytotoxic/mature phenotype, characterized by *KLRB1 (CD161), FCGR3A (CD16)*, and *CXCR4*, evenly distributed across conditions. **Cluster 2**, with the highest percentage of CD123-CAR-NK and lowest of NT-NK cells, comprised an activated cytokine-secreting state enriched for *CSF2 (GM-CSF), CCL1 (I-309), IFNG, IL3*, and *IL1A*. **Cluster 3**, slightly increased in CD123-CAR-NK cells, contained stressed/exhaustion-leaning cells expressing ER stress and apoptotic programs: *DDIT3 (CHOP), TRIB3, TSC22D3, SESN2, BBC3 (PUMA)*, and *CHAC1* (Fig. 3B, Table S1). **Cluster 4** represented an adaptive/inhibitory phenotype, characterized by *LAIR1, KIT*, and immune-regulatory genes *IFITM3* and *GRAP2*. This cluster accounted for the lowest percentage of CD123-CAR-NK and highest of NT-NK cells. Overall, CD123-CAR NK cells were enriched in activated/cytokine-producing and exhausted clusters, whereas CD33-CAR NK cells resembled non-transduced NK cells.

As the percentage of CAR-positive cells varied (Fig. S5A), a second analysis examined only CAR-positive cells (Fig. 3C, D). Within this subset, three transcriptional states emerged (Fig. 3C). **Cluster C1** represented baseline cytotoxic CAR-NK cells, expressing *FCGR3A (CD16), CXCR4, KLRB1 (CD161), MAF*, and *RORA*; CD123-CAR-NK cells exhibited higher percentages of this cluster. **Cluster C2**, a hyperactivated effector population with high *IFNG, CSF2, IL3, IL1A, CCL1, LTA*, and *TNFSF11*, was more pronounced in CD33-CAR-NK cells. **Cluster C3** corresponded to a dysfunctional/exhausted state, enriched for histone genes, apoptotic mediators like *BBC3*, and stress/inflammatory regulators including *EBPD* and *ARRDC3* (Fig. 3D, Table S2). CD123-CAR NK cells displayed a higher proportion of cytotoxic and exhausted states, consistent with a tissue-adaptive phenotype where *CXCR4* upregulation may promote retention within *CXCL12*-rich AML niches. Conversely, CD33-CAR NK cells were enriched in the hyperactivated/talker state, potentially reflecting a highly cytokine-secreting but less persistent effector phenotype. Differentially expressed genes (Fig. 3E, Table S3) and gene set enrichment analysis (Fig. 3F, Table S4) revealed a shift in CD123-CAR NK cell state compared with CD33-CAR NK cells, characterized by reduced acute cytokine effector function and increased enrichment of proliferation- and cytoskeleton-associated programs.

CD123-CAR-NK cells show high cytotoxic efficacy against patient derived primary AML cells in vitro and in vivo

To validate results in a clinically representative AML model, CAR products were challenged with primary AML cells in vitro (Fig. 4A and B) and in vivo (Fig. 4C-H). CD33 and CD123 surface expression were measured in all samples prior to cytotoxicity assessment or injection (Fig. 4A). In vitro cytotoxicity was

assessed using cells from three AML patients (F#1-#3) at an E:T ratio of 1:1 and 4 h of co-incubation with CD123-/CD33-CAR-NK cells (Fig. 4B). Patient #F1 showed 12% lower CD33 than CD123 expression (63,0% vs. 75,0%), patient #F3 showed 18% lower (65,9% vs. 83,9%), and patient #F2 showed higher CD33 than CD123 (89,1% vs. 78,4). CD123-CAR-NK cells significantly decreased viable AML cells in all samples compared to NT-NK cells. CD33-CAR-NK cells showed increased specific lysis in patient #F2.

For in vivo validation, a PDX model in NSG mice used $1x10^6$ AML cells from patient T#1, carrying a NPM1 t(3;7) (q25;q11.2) mutation. Donor cells exhibited 95% surface expression of both CD33 and CD123 (Fig. 4A). After 96 days, mice were grouped by bone marrow tumor load (Fig. S3B) and treated with CAR- or NT-NK cells or were left untreated. Residual tumor and NK cells were monitored or analyzed at endpoint (gating strategy, Fig. S3A). In peripheral blood, NK cell expansion was observed in all treated groups (Fig. 4D). However, residual CD33 and CD123 positive AML cells in peripheral blood were significantly reduced only in the CD123-CAR-NK group (Fig. 4E). No significant decrease in bone marrow tumor cells occurred; only a tendency for reduction of CD33 positive cells appeared in the CD123-CAR-NK group. Notably, residual CD33 positive AML cells in bone marrow were 75% higher than CD123 positive cells (Fig. 4F). In spleen and liver, significant AML reduction was observed in CD123-CAR-NK mice but not the CD33-CAR-NK group (Fig. 4G-H). The same applied to the spleen, where no tumor burden reduction was recorded for the CD33-CAR-NK group (Fig. 4G). Together, the AML PDX model data confirmed the high antileukemic superiority of CD123-CAR-NK cells compared with CD33-CAR-NK cells.

Histological analysis reveals significantly decreased tumor burden without histopathological alterations after CD123-CAR-NK cell treatment

Histological analysis of PDX model bone marrow, spleen, liver, and lungs was conducted at endpoint using hematoxylin and eosin (H&E), anti-human CD33, and anti-human CD123 staining. Samples were scored blindly from 0 (negative/minimal) to 4 (prominent/severe) based on positive cell counts. Generally, no histopathological changes occurred beyond tumor infiltration. In the UT- and NT-NK cohorts (Fig. 5A), bone marrow infiltration was severe, to the point of minimal hematopoiesis (Fig. 5B). Immunohistochemistry revealed consistent CD33 positivity (Fig. 5C) and heterogeneous CD123 expression (Fig. 5D). The spleen showed diffuse infiltration with marker expression resembling the bone marrow. Similar patterns occurred in the CD33-CAR-NK cohort; however, $CD33^+$ AML cells in lungs and liver decreased compared to $CD123^+$ cells, suggesting mild antitumor effects (Fig. 5C, D). The CD123-CAR-NK cohort showed the most pronounced effects, with significantly decreased tumor infiltration in lungs and liver (Fig. 5B), though infiltration remained prominent in bone marrow. $CD123^+$ tumor cells were significantly reduced in all organs compared to the CD33-CAR-NK group (Fig. 5D). CD33 staining showed no liver or lung infiltration, mild splenic infiltration, and $CD33^+$ bone marrow cells (Fig. 5C). Histological analysis confirmed flow cytometry observations (Fig. 4E-H), demonstrating that CD123-CAR-NK cells were more efficient than CD33-CAR-NK cells at eliminating AML in this PDX model.

Large-scale production of CD123-CAR-NK cells is feasible and highly efficient

To evaluate the upscaling potential of CD123-CAR-NK cells for clinical CAR-NK therapy, we produced these cells using the CliniMACS Prodigy system on small- and large-scale (Fig. 6A). Small-scale production involved NK cells isolated via CliniMACS Prodigy and expanded using a manual protocol. Vector copy numbers were measured to ensure safe genomic integration and assess transduction efficacy, yielding low values of ~ 2.5 for both small-scale (SmS) and large-scale (PROD) cells (Fig. 6B). CAR surface expression, viability, and CD69, CD57, and NKG2A expression were measured via flow cytometry, showing comparable values between SmS and PROD, except for viability (91.5% vs 85.0%, respectively) (Fig. 6C). Expansion rates were monitored during quality control (Fig. S2A, B), alongside representative CAR expression on day 14 (Fig. S2D). We observed a tendency for decreased expansion in PROD compared to SmS, and SmS compared to NT-NK cells. In vitro functionality was assessed using OCI-AML2^{CD123+} cells after 4 h and 20 h (Fig. 6D); both products showed high cytotoxic potential at 1:1 and 5:1 E:T ratios.

To investigate PROD cells for clinical use, we performed single-cell RNA-seq after co-incubation with OCI-AML2^{CD123+} for 4h. Differentially expressed genes (Fig. 6E, Table S5) and gene set enrichment analysis (Fig. 6F, Table S6) revealed that upscaling during Prodigy-based manufacturing did not negatively influence the cells, but induced a shift into a regulated, tissue-adaptive state with enhanced cytokine sensitivity, survival signaling, and migratory capacity compared to SmS production. Subsequently, both products were tested in an OCI-AML2^{CD123+} xenograft model with 10x10^6 CAR-expressing NK cells administered on day 3 (Fig. 6G). BLI (Fig. 6H) and total flux (Fig. 6I) demonstrated reduced tumor load in both CAR-NK groups versus NT-NK and untreated controls. Endpoint analysis of bone marrow tumor load on day 21 showed a significant decrease in AML burden in both CD123-CAR-NK groups (Fig. 6J). These data suggest that upscaling CD123-CAR-NK cells is feasible, maintains functional antileukemic capacity, and confers additional benefits, including increased migratory and tissue-adaptive status.

Discussion

Whilst CAR-NK cell therapy is a promising treatment for AML, identifying a suitable target antigen has been challenging.[6] Several ongoing clinical studies are testing one or two of the major known AML antigens: CD33, CD123, CLEC12A (CLL-1), NKG2D-L, CD70, CD7.[16] Most CAR-NK AML research focuses on target validation[12], co-stimulatory domains[17], IL-15 engineering[18], or comparing CAR-T with CAR-NK cells[19]. This study presents an in-depth characterization and comparison of two AML-specific CAR-NK cell products targeting CD123 or CD33 in fully matched in vitro and in vivo settings to support upscaling of the most promising CAR-NK cell preparation for clinical translation. The CAR ratio, expansion, and cytotoxic efficiency align with previous findings.[10,12,20] However, CD123-CAR-NK cells outperformed CD33-CAR-NK cells in vitro and in vivo, resulting in fewer high-tumor-burden regions, less leukemic bone

marrow invasion, and increased survival. Notably, CD33-CAR-NK cells reduced AML load preferentially in a low-burden in vivo model, but not in highly proliferating disease stages, consistent with our data.[10,12,20]

As other AML cell lines are sensitive to primary NK cells[12], we engineered CD33-expressing OCI-AML2 to express high CD123 levels to compare CAR-NK products against a uniform target. Since this modification might affect cytolytic capacity, results were validated using primary AML samples. In vitro cytotoxicity against two of three primary AML donors was significantly higher with CD123-CAR-NK cells, despite expression of both targets on ≥ 60% of cells. This may result from CD123's higher functional antigen density, which efficiently surpasses NK activation thresholds.[21] In vivo anti-leukemic efficiency against primary AML was observed only for CD123-CAR-NK cells, despite comparable target expression (≥ 90%), consistent with prior results. In the PDX model, no bone marrow tumor reduction was achieved, possibly due to high tumor burden at injection (Ø 42%), strong AML homing[22], and the immune suppressive bone marrow niche[23]. Nevertheless, these data highlight the robustness of CD123-CAR-NK cell products in eliminating AML under the tested in vivo conditions.

To unravel mechanisms allowing higher tumor-targeting, deep single-cell RNA sequencing was performed on the total and CAR-positive NK populations. CD123-CAR-NK cells comprised a larger percentage of the active/cytokine cluster than CD33-CAR-NK and NT-NK cells. Comparing CAR-positive cells, CD123-CAR-NK cells showed more cytotoxic/killer NKs with increased *CXCR4* expression. This tissue-adaptive phenotype enables retention within *CXCL12*-rich AML niches[24] and could explain favorable in vivo results. Additionally, clusters were scored against NK1-3 subsets from healthy human blood[25]; cluster 1 resembled NK3 (HCMV-driven adaptive NK cells) and cluster 2 resembled NK1 (canonical CD56dim) (Fig. S5B). However, differences in culture and NK sources limit comparability.

After establishing our CD123-CAR NK cell product as a valuable AML therapy, the next step for clinical translation is large scale manufacturing in sufficient quantities, which is a challenging process. We have already established a feeder cell-free approach based on cytokine stimulation with the CliniMACS Prodigy system for our CD33-CAR-NK cell product[10], which has now been validated with CD123-CAR-NK cells in the present work. After 14 days of cultivation, we achieved a 16.5-fold expansion, yielding a total of $1.65x10^9$ highly functional CAR-NK cells, which would be sufficient for treating 2–20 patients, depending on dosing regimen.[26]

Regarding biological differences in target efficacy, CD33 is expressed on NK cells and belongs to the Siglec family, which links to inhibitory signaling and may impair NK cell function.[27] Additionally, CD33-CAR-NK cells might exhaust faster due to high bone marrow CD33 expression.[8] Conversely, CD123 shows stable surface expression, lacks inhibitory motifs, is absent on NK cells, and has lower off-target expression, facilitating sustained activation and effective tumor control in preclinical models.[9] However, intrinsic biological differences and CAR affinity affect immunological synapse formation, influencing the killing capacity of CAR-NK cell products.[28] Our findings highlight that target biology and antigen density are critical determinants of CAR-NK cells efficacy against AML.

Although targeting CD33 proved less promising than CD123, CD33 remains a key AML target. Reported high toxicities from myeloablation after CD33 targeting are largely based on CAR-T cell studies, which show increased persistence and expansion compared to CAR-NK cells.[29] The slower expansion rate of NK cells, combined with high cytolytic potential, can reduce myeloablation risks in CD33 targeting while maintaining efficiency, as shown in clinical trials.[30] Additionally, off-tumor effects can be circumvented with restricted targeting via gated CARs.[31] Generally, CD123 is considered a safer AML target due to lower expression on HSCs,[9] with four ongoing early phase CAR-NK cell trials (NCT06201247, NCT05574608, NCT06006403, NCT06690827)[32] and one study showing high safety of CD123-CAR-NK cells with an 80% response rate in 5 patients.[33]

Several approaches can enhance CAR-NK efficacy and ensure fast LIC eradication.[34] For example, deleting cytokine checkpoints like CIS, increases (CAR-)NK activation[35], inhibitory receptors like NKG2A can be downregulated, increasing CD33-CAR-NK killing[36], and persistence can be improved by local autocrine IL-15 secretion, preventing systemic side effects[37]. Another aspect of efficient AML targeting is multi-antigen or logic-gated strategies addressing heterogeneity and escape. Bivalent CD33-CAR-NK products show promising results while sparing HSCs via NOT gates for protective antigens like endomucin.[38] Several preclinical and early clinical studies are evaluating bispecific or dual targeting of CD123 and CD33.[9,39–41]

Overall, this study demonstrates that the CD123-CAR-NK cell product mediates superior cytolytic capabilities compared to the CD33-CAR-NK cell product across all tested models. Additionally, a feeder-cell-free upscaling method was developed and evaluated for CD123-CAR-NK cell production, which is crucial for clinical translation. Our comparative analysis, utilizing both in vitro and in vivo experimental frameworks, yields crucial insights into the signaling pathways of CAR-NK cells that contribute to the observed enhanced functionality of the CD123-CAR CAR-NK cell product.

Abbreviations

AML	Acute Myeloid Leukemia
BaEV	Baboon EnVelope
CAR	Chimeric Antigen Receptor
CLL-1	CLEC12A
CRS	Cytokine Release Syndrome
E:T	Effector:Target
H&E	Hematoxylin and Eosin

HSPC	Hematopoietic Stem and Progenitor Cell
LIC	Leukemia Initiating Cells
NK	Natural Killer
NT	Non-Transduced
PBMC	Peripheral Blood Mononuclear Cell
PDX	Patient-Derived Xenograft
PEI	PolyEthylenImine
PROD	PRODigy (large-scale)
scFv	single chain variable Fragment
SmS	Small-Scale
UT	Untreated

Declarations

Authorship Contributions

EU designed the research, analyzed and interpreted the data, wrote and revised the manuscript and supervised the study. CC and CL analyzed and interpreted the data and revised the manuscript. FG performed the experiments, analyzed and interpreted data, wrote the original draft of the manuscript and edited the manuscript. LK, SR, PW, JS, HMR, NL, LQF, IGM, SM, JKo, JKu performed experiments, analyzed and interpreted data. CKK, DL, CB, KR produced, analyzed and interpreted bioinformatic data. CC, SH, JHK, UK, OP, NM revised the manuscript.

Disclosure of Conflicts of Interest

E.U. has no COIs directly related to this manuscript. E.U. has a sponsored research project with Gilead and BMS. No competing financial interests exist for the remaining authors. SM, JKo, JKu and CZ are employees of Miltenyi Biotec.

Data Sharing Statement

The data generated in this study are available upon request from the corresponding author. The Single Cell Sequencing data generated in this study are publicly available in Gene Expression Omnibus (GEO) under accession number GSE337768 (reviewer token: mdgbyaogpdcbrad). The scripts used for the spatial data analysis are publicly available on GitHub: https://github.com/fraunhofer-izi/Gierschek_et_al_2026. Other data generated in this study are available within the article and its supplementary data files. Schematic figures were created with BioRender.com (Graphical abstract; Fig. 1A, B, E, G; Fig. 2A, E; Fig. 4C; Fig. 6A, G).

Acknowledgement

We thank Katja Stein, Nawid Albinger, Jan Habermann, Alina Moter, Lea Knapp, Tobias Bexte and Lisa Marie Reindl for their continuous support and scientific input. We thank Kristina Götze, Thomas Oellerich, Christian Brandts & Team for the seamless organization and providing of patient's samples. We also thank the supportive team at the animal facility at Georg Speyer Haus Frankfurt. This project was partly funded by the German Cancer Aid (NK fit against AML) and the German Cancer Consortium (DKTK) Joint Funding Program (NoviCARAZA) awarded to EU and CL.

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Figures

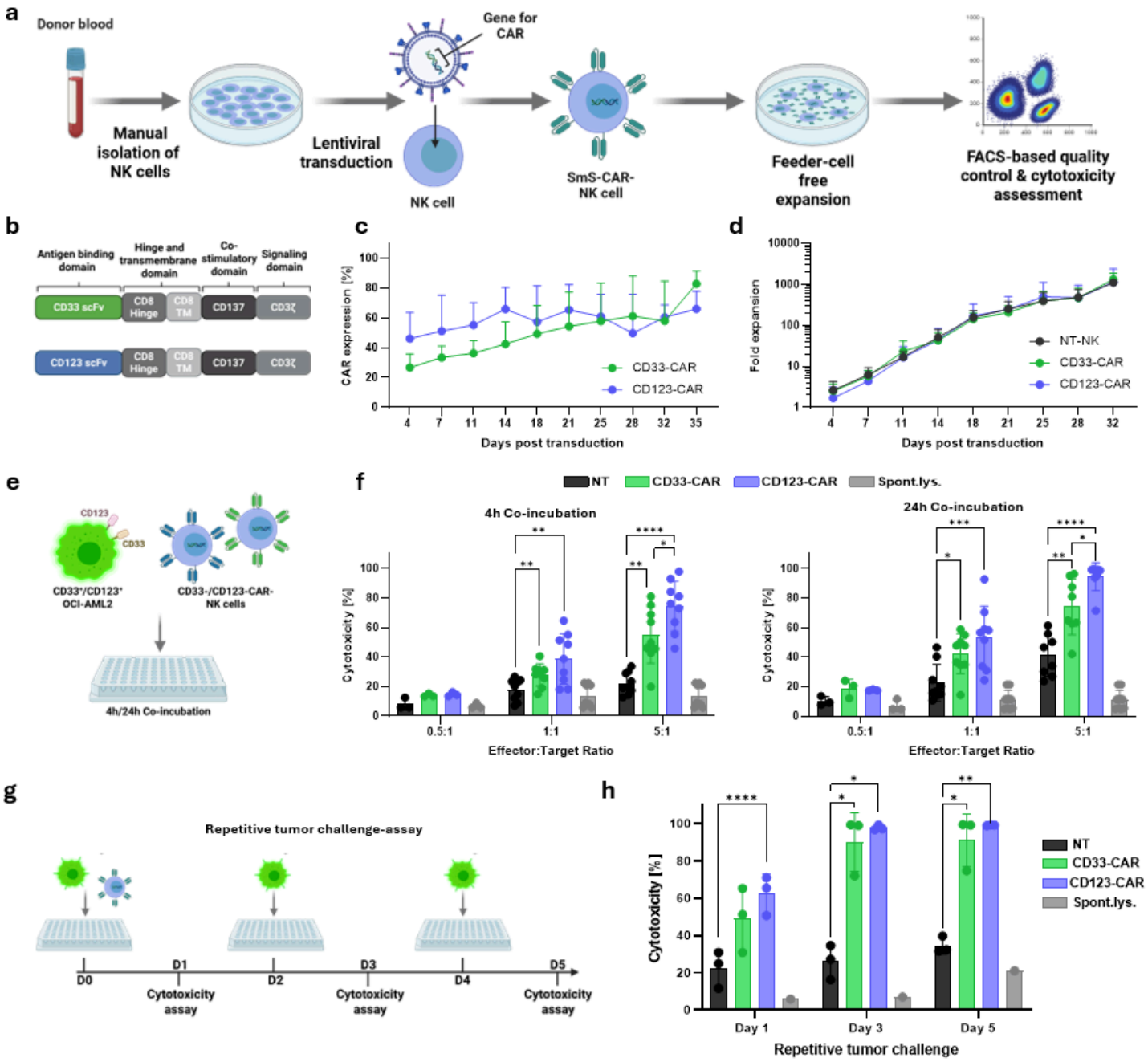


Figure 1

CD123- and CD33-CAR-NK cells demonstrate anti-leukemic efficacy in vitro. A. *Overview of the production process of lentivirally transduced CAR-NK cells from isolation to quality control.* ***B.*** *Illustration of second-generation CAR constructs used to produce either CD123- or CD33-CAR-NK cells.* ***C.*** *Percentage of CAR expressing cells in culture over time (n=6).* ***D.*** *Fold expansion of CAR-NK cells and NT-NK cells over time (n=4).* ***E.*** *Illustration of cytotoxicity assay against CD33/CD123 double positive OCI-AML2^{CD123+}.* ***F.*** *In vitro cytotoxicity of CAR-NK cells against OCI-AML2^{CD123+} after 4 hours (left) and 24 hours (right) (n=2-8).* ***G.*** *Illustration of "repetitive tumor challenge assay".* ***H.*** *In vitro cytotoxicity of CAR-NK cells against OCI-*

*AML2^{CD123} after repetitive tumor challenge at effector to target ratio 1:1 (n=3). Statistical analysis was carried out by two-way ANOVA. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001*

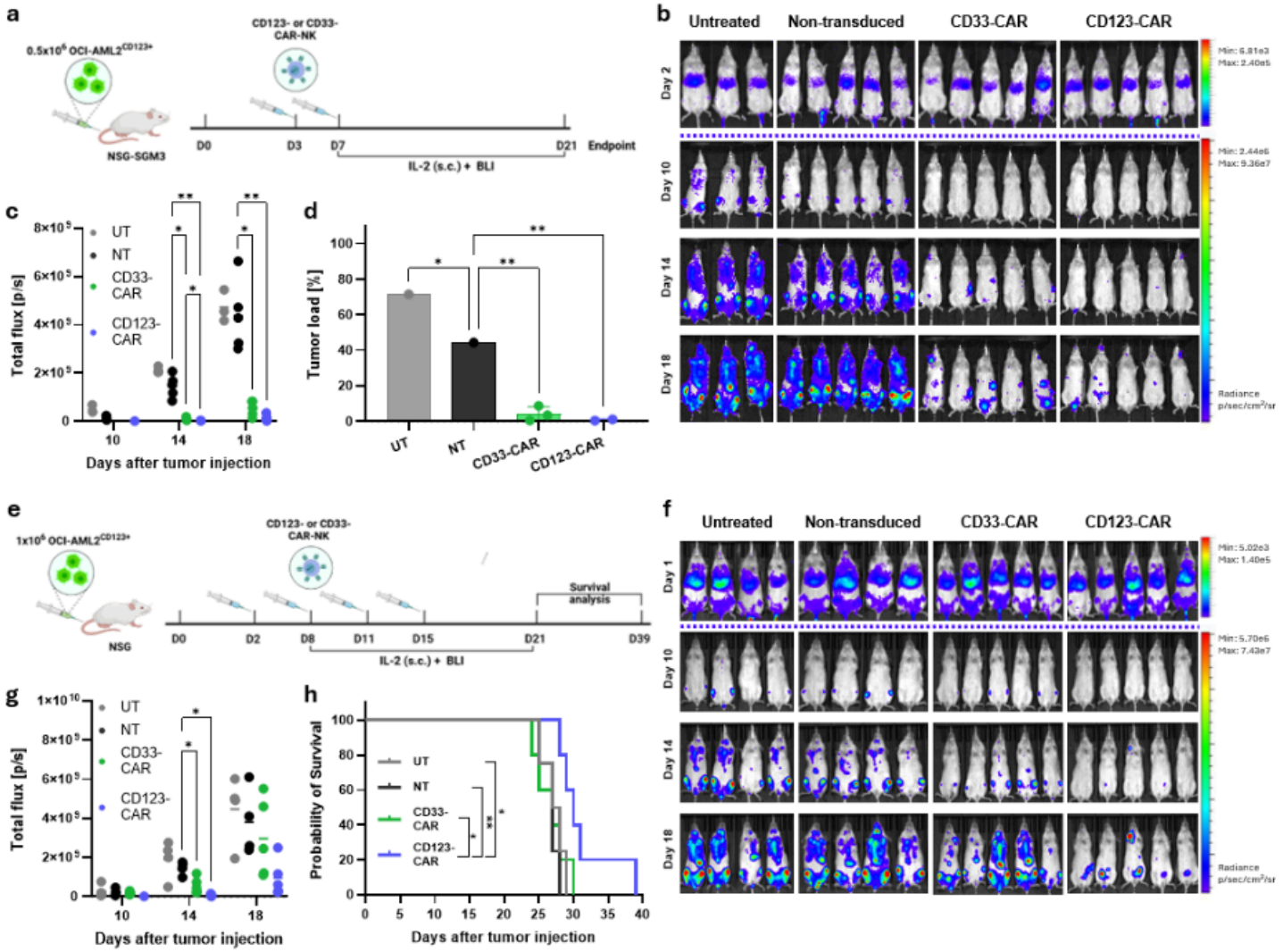


Figure 2

CD123- and CD33-CAR-NK cells demonstrate systematic anti-leukemic efficacy in vivo, whilst only CD123-CAR-NK cells increase survival significantly. A. *Schematic representation of treatment protocol for xenograft mouse model used for B-D.* ***B.*** *Bioluminescence imaging and corresponding total flux* ***C.*** *of differently treated groups (n=3-5) over time, including grouping on day 2 after tumor injection.* ***D.*** *Percentage of residual OCI-AML2^{CD123} cells in the bone marrow of different groups determined by flow cytometry on day 21 post tumor injection (n=1-3).* ***E.*** *Schematic representation of treatment protocol for xenograft mouse model used for F-H.* ***F.*** *Bioluminescence imaging and corresponding total flux* ***G.*** *of differently treated groups (n=4-5) over time, including grouping on day 1 after tumor injection.* ***H.*** *Probability of survival over time of differently treated groups (n=4-5). Statistical analysis was carried out by Log-rank (Mantel-Cox) test (H), one-way ANOVA (D) or two-way ANOVA (C, G). * P ≤ 0.05, ** P ≤ 0.01*

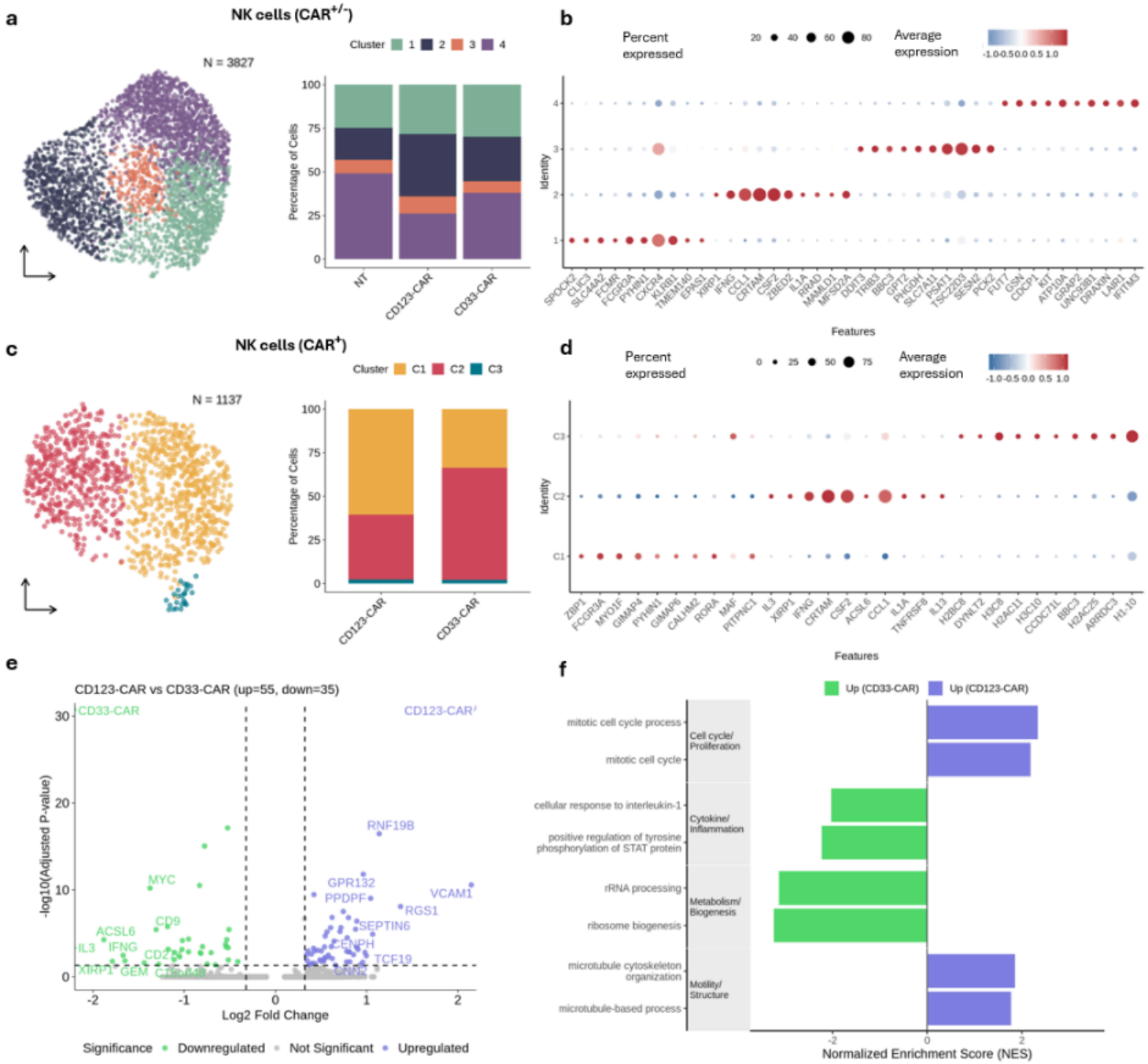


Figure 3

Single-cell sequencing reveals CD123-CAR+ NK cells have a more cycling, tissue-adaptive phenotype compared to CD33-CAR+ NK cells. (A) *Schematic representation of in vitro co-culture assay used prior to fixation and 10xFlex sequencing. NT=Non-transduced.* ***(B)*** *Clustering of all NK cells (CAR+/-) and corresponding cluster distribution.* ***(C)*** *Top 10 most distinguishing genes expressed between four identified CAR(+/-) NK clusters. Gene expression was analyzed using the two-sided Wilcoxon rank-sum test with Bonferroni adjustment. Dot color reflects scaled expression (Z-score), and dot size represents the percentage of expressing cells.* ***(D)*** *Clustering of only CAR+ NK cells and corresponding cluster distribution.*

*(**E**) Top 10 most distinguishing genes expressed between four identified CAR+ NK clusters. **(F)** Up- and downregulated genes of CAR+ CD123-CAR-NK cells compared to CD33-CAR-NK cells. **(G)** GSEA-derived Normalized Enrichment Scores highlighting the most significantly upregulated and downregulated cellular pathways. GSEA = gene set enrichment analysis.*

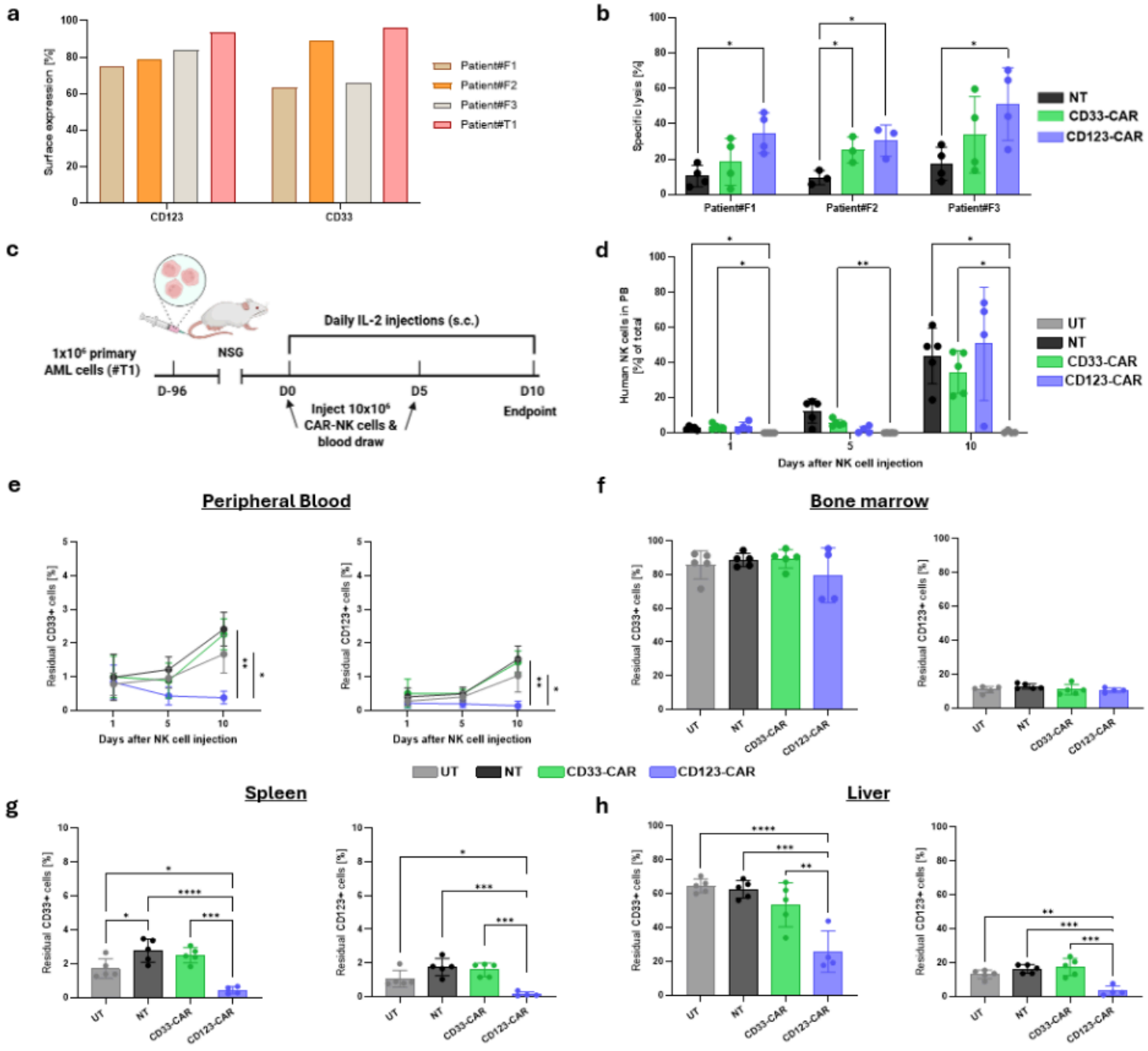


Figure 4

CD123-CAR-NK cells show high cytotoxic efficacy against patient-derived primary AML cells in vitro and in vivo. A. *Surface expression of CD123 and CD33 on primary AML samples used for in vitro cytotoxicity assessment (F#1-#3) and in vivo PDX model (T#1).* ***B.*** *In vitro cytotoxicity of CAR-NK cell products against primary AML samples after 4 hours of co-incubation at effector to target ratio 1:1 (n=3-4).* ***C.*** *Schematic*

representation of treatment protocol for PDX mouse model with donor #T1 (NPM1mut, t(3, 7). ***D.*** *Percentage of NK cells in peripheral blood of differently treated mice on day 1, 5, and 10.* ***E.*** *Percentage of residual CD33+ (left) and CD123+ (right) cells in peripheral blood (PB) of differently treated mice on day 1, 5, and 10.* ***F-H:*** *Percentage of residual CD33+ (left) and CD123+ (right) cells in the bone marrow* ***(F)****, spleen* ***(G)*** *and liver* ***(H)*** *at endpoint. Statistical analysis was carried out by one-way ANOVA (F-H) or two-way ANOVA (B, D, E). * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001*

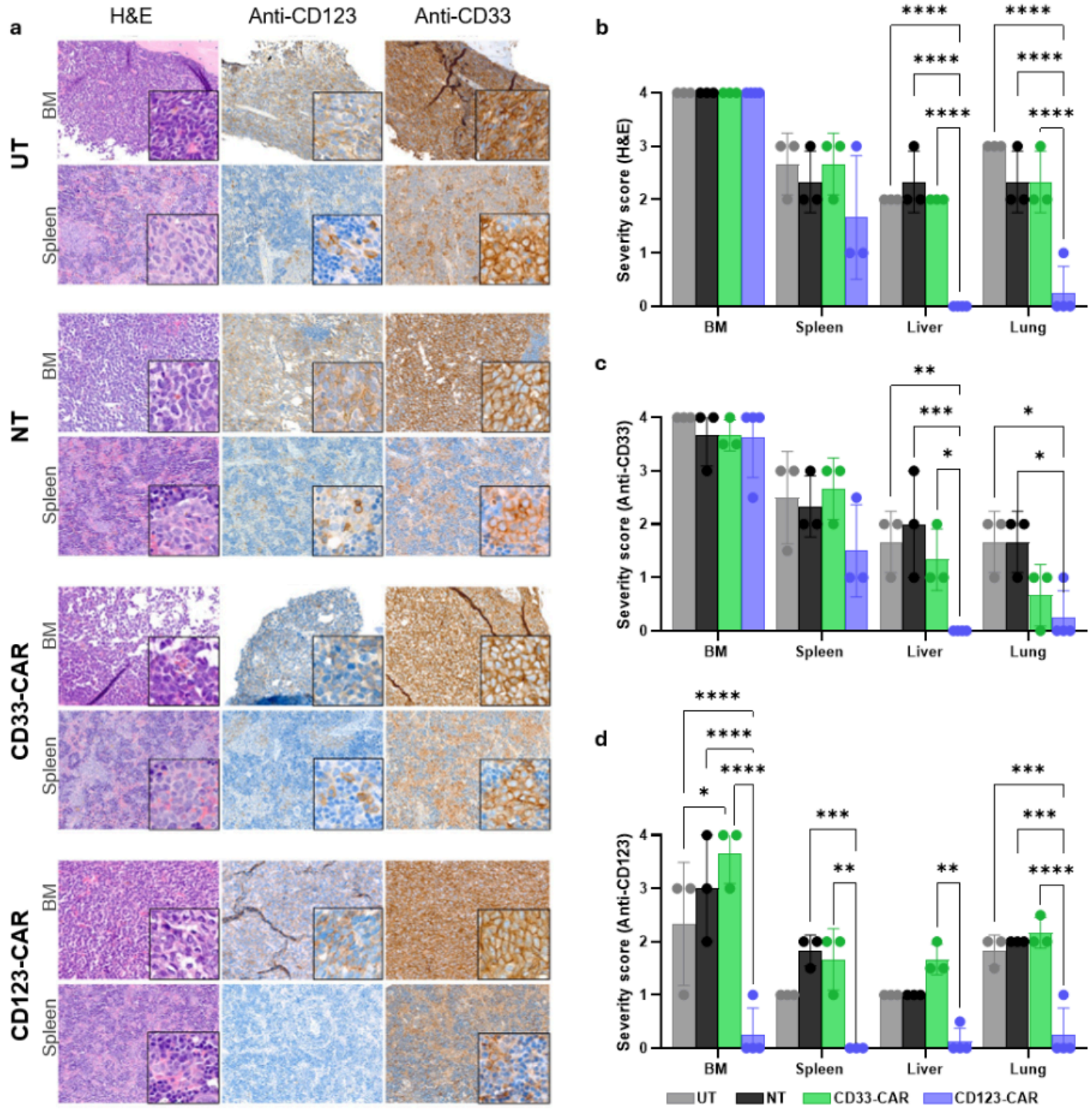


Figure 5

CD123-CAR-NK cells significantly decrease tumor infiltration of organs in a PDX mouse model. A. *Histological analysis with hematoxylin and eosin (H&E), anti-human CD33 and anti-human CD123 staining of bone marrow (BM) (40x) and spleen (20x) at endpoint of untreated mice (n=3), mice treated with non-transduced (NT)-NK cells (n=3) or mice treated with CD33- or CD123-CAR-NK cells (n=3 and n= 4).* ***B-D:*** *Tumor infiltration-* ***(B)****, CD33-* ***(C),*** *and CD123-* ***(D)*** *severity score in bone marrow, spleen, liver and lung of animals untreated or treated with NT-, CD33-CAR, or CD123-CAR-NK cells. Severity score = 0: negative or minimal; 1: low; 2: mild; 3: moderate; 4: prominent. Bars show mean values with corresponding standard deviation. Statistical analysis was carried out by two-way ANOVA (B-D). * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001*

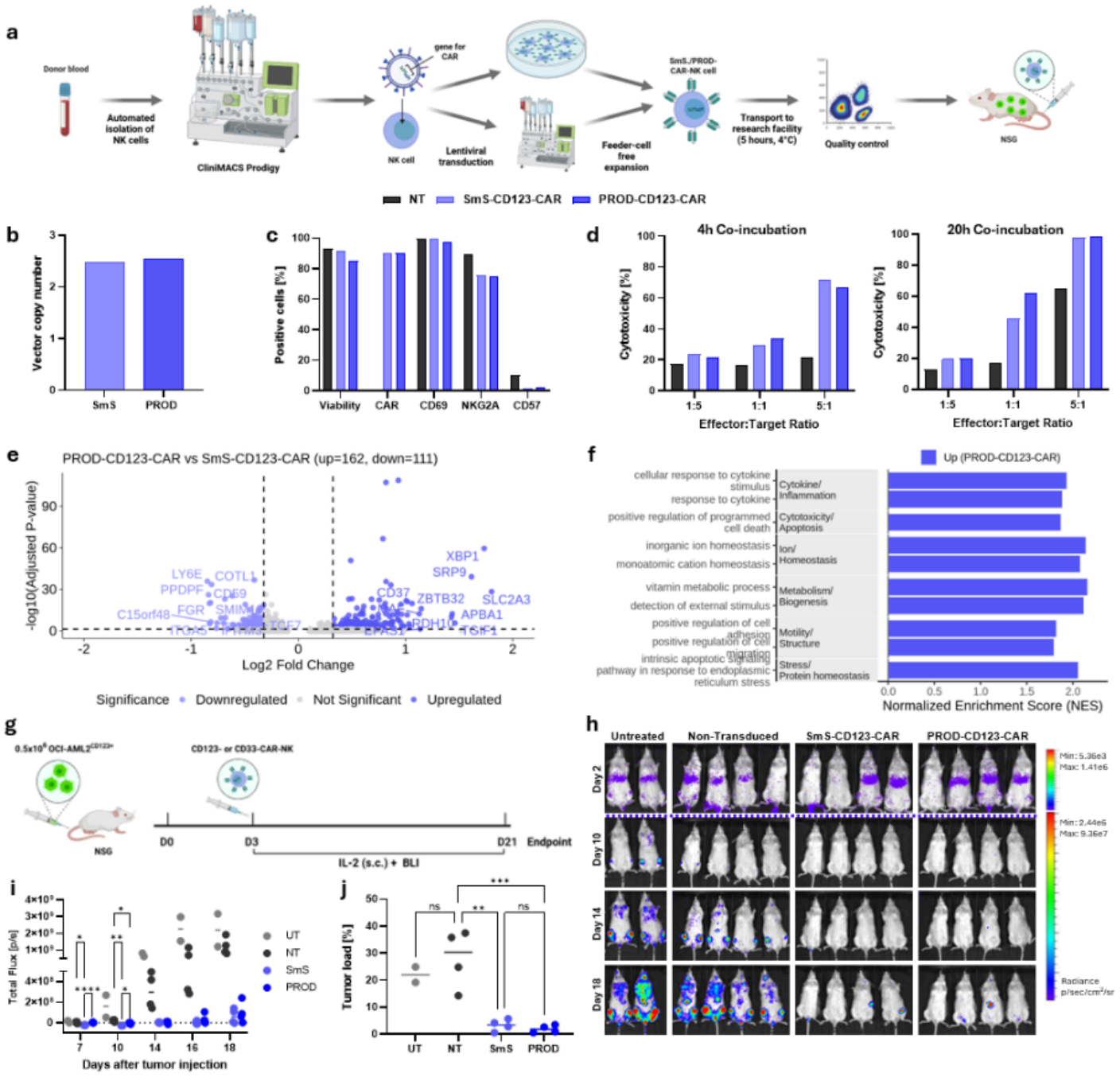


Figure 6

CliniMACS Prodigy-produced CD123-CAR-NK cells show similar characteristics and high cytotoxic capacity in vitro and in vivo against OCI-AML2^{CD123} cells compared to small-scale production. A. *Overview of the production process of CAR-NK cells using the Prodigy system for small-scale (SmS) and large-scale (PROD) production.* ***B.*** *Vector copy number of small- and large-scale produced CAR-NK cells.* ***C.*** *Marker expression of produced SmS- and PROD-CD123-CAR-NK cells after transport for 5 hours at 4°C* ***(n=1). D.*** *In vitro cytotoxicity of CAR-NK cell products against OCI-AML2^{CD123} after 4 and 20 hours of co-incubation at different effector to target ratios after transport* ***(n=1). E.*** *Differentially expressed genes between PROD-CD123-CAR+ NK and SmS-CD123-CAR+ NK cells (absolute fold change > 1.25, adjusted p-value < 0.05).* ***F.*** *Gene set enrichment analysis (GSEA)-derived Normalized Enrichment Scores highlighting the most significantly upregulated cellular pathways. No significantly downregulated pathways were identified.* ***G.*** *Schematic representation of the treatment protocol for the xenograft mouse model used for H-J.* ***H.*** *Bioluminescence imaging and corresponding total flux* ***(I)*** *results of differently treated groups over time* ***(n=2-4). J.*** *Percentage of residual OCI-AML2^{CD123} cells in bone marrow of different groups determined by flow cytometry* ***(n=2-4).***

Supplementary Files

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- SupplementIndepthcharacterizationofCD123CARNKcellsEvelynUllrichGierscheketal2026.docx