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Received: May 30, 2026.

Accepted: October 9, 2026.

Citation: Makiko Suga, Shuhei Kida, Yo Mizutani, Yosuke Kogue, Yuta Yamaguchi, Shunya Ikeda, Ryuhei Kawamoto, Mana Tachikawa, Tsubasa Seto, Hiroto Takekawa, Yuto Maehara, Kumi Shibata, Kazuhito Tsutsumi, Hiraku Murakami, Emiko Mizuta, Kyoko Yoshihara, Satoshi Yoshihara, Hirohisa Nakamae, Takafumi Nakao, Megumu Inoue, Shuji Ueda, Satoru Kosugi, Yasutaka Ueda, Hisashi Kato, Shinsuke Kusakabe, Hidenori Kasahara, Akihisa Hino, Jiro Fujita, Tomoaki Ueda, Michiko Ichii, Kentaro Fukushima and Naoki Hosen. Cord blood-derived natural killer cells engineered with a novel, high-affinity chimeric antigen receptor can eliminate acute myeloid leukemia cells with minimal C-type lectin-like molecule-1 expression.

Haematologica. 2026 Oct 15. doi: 10.3324/haematol.2026.301327 [Epub ahead of print]

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Cord blood-derived natural killer cells engineered with a novel, high-affinity chimeric antigen receptor can eliminate acute myeloid leukemia cells with minimal C-type lectin-like molecule-1 expression

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Authors' contributions

MS, SKi and NH designed the research study, performed the experiments, analyzed the data, and wrote the paper; YMi, YK, SI, RK, MT, TS, HT, YMa, KS, KT, HM, EM, YY, KY, SY, HN, TN, MIn, SU, SKo, YU, HKat, SKu, HKas, AHi, JF, TU, Mlc, and KF performed the experiments. MS and SKi contributed equally as co-first authors.

Running heads

Novel, highly sensitive CLL-1 CAR NK cells

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Data-sharing statement.

Datasets and detailed protocols supporting the findings of this study are available from the corresponding author upon reasonable request. Please contact Dr. Naoki Hosen at hnaoki@bldon.med.osaka-u.ac.jp.

Acknowledgments

We wish to thank the Hyogo Cord Blood Bank and Kinki Cord Blood Bank for providing

cord blood samples. We thank K. Terasaki and Y. Takashima for their technical assistance. MS is a PhD candidate at the University of Osaka Graduate School of Medicine, and this work was submitted in partial fulfillment of the requirements of the PhD.

Funding

This work was supported in part by the Japan Agency for Medical Research and Development (AMED) (22cm0106381, 24bk0104170, and 25ama221341 to N.H.), Japan Society for the Promotion of Science (JSPS) KAKENHI (JPJSBP120237202, JP21K19508, JP23K18300, JP23H04781, JP25K22633, and JP20H03710 to N.H.), and grants from the Takeda Science Foundation (to N.H.).

Declaration of interests

NH has applied for a Japanese patent entitled “Antibodies against CLL-1” through the University of Osaka Office for University–Industry Collaboration. The other authors declare no competing financial interests.

Abstract

CAR NK cell therapy is expected to be developed to treat acute myeloid leukemia (AML). C-type lectin-like molecule 1 (CLL-1) is a candidate target. However, AML cells with minimal CLL-1 expression (CLL-1^{dim}) may not be eliminated by CAR NK cells. Here we demonstrate that cord blood (CB)-derived NK cells transduced with a novel high-affinity CAR that recognizes CLL-1 can eliminate CLL-1^{dim} AML cells. A newly established anti-CLL-1 monoclonal antibody (mAb) designated as 2-23 exhibits a higher affinity for CLL-1 than the widely used anti-CLL-1 mAb M26. CB-derived NK cells transduced with a CAR derived from 2-23, but not M26, killed CLL-1^{dim} AML cells *in vitro*. Additionally, treatment with 2-23 CAR NK cells, but not M26 CAR NK cells, significantly enhanced the survival of the mice transplanted with CLL-1^{dim} AML cells *in vivo*. Furthermore, in AML patient-derived xenograft models, the 2-23 CAR NK cells expanded robustly, persisted, and eradicated AML cells.

Introduction

The major limitations of chimeric antigen receptor (CAR) T-cell therapy are the lengthy manufacturing process and the high cost. Cord blood (CB)-derived CAR NK cells can be produced as allogeneic, off-the-shelf products for immediate clinical use¹. CAR NK cells for multiple patients can be produced from a single CB unit¹, which could reduce the cost of CAR NK cell therapy compared to CAR T-cell therapy. In addition, allogeneic CB-derived NK cells may have an advantage in that they are not exhausted, whereas autologous T cells are frequently dysfunctional after multiple lines of therapy. Clinical trials of CB-derived CAR NK cells targeting CD19 have demonstrated a significant anti-tumor effect^{2,3}.

Acute myeloid leukemia (AML) is a hematological malignancy with a poor prognosis. Patients who cannot be cured with chemotherapy undergo allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, many of these patients cannot be cured even with allo-HSCT. In particular, fewer than 20% of patients with AML who undergo allo-HSCT during the non-remission stage or who relapse after allo-HSCT achieve long-term survival, primarily due to disease relapse⁴. Therefore, additional therapies for patients with AML are urgently needed. Recently, several new AML treatments have been developed, including CD47-blocking agents⁵, CD70 antibodies⁶,

and targeted small molecules. Cellular therapies, such as CAR T-cell or NK cell therapy, are expected to be effective AML treatments. CAR NK cell therapy is a novel AML therapy being developed using several different target antigens ⁷⁻¹⁰. In addition to CD33, CD123, and CD70, C-type lectin-like molecule 1 (CLL-1) is a promising target ¹¹.

CLL-1, also known as CD371, CLEC12A, or MICL, is expressed in AML leukemic stem cells (LSCs) in many patients. In contrast, CLL-1 is not expressed in normal hematopoietic stem cells (HSCs) ^{12,13}. CLL-1 is not expressed in T cells, B cells, natural killer (NK) cells, red blood cells, or non-hematopoietic tissues; however, it is expressed in almost all granulocytes and monocytes and their progenitor cells. Preclinical studies and some clinical trials have demonstrated that CAR T-cell therapy targeting CLL-1 can reduce AML burden ¹⁴⁻¹⁶. However, it can cause severe and continuous neutropenia. Due to the potential for adverse side effects, CLL-1 CAR T-cell therapy is being investigated as a bridge to allo-HSCT, rather than as a stand-alone therapy¹⁷. CAR NK cells targeting CLL-1 may also be useful in eradicating AML stem cells prior to allogeneic hematopoietic stem cell transplantation.

CLL-1 expression varies in AML. CLL-1 is detected in CD34⁺CD38⁻ LSC-enriched AML cell populations, but its antigen density varies widely, ranging from high to low ¹⁸. CLL-1 CAR T cells have demonstrated anti-leukemic activity against AML

cells with high CLL-1 expression^{14,19-21}. However, CLL-1 CAR T cell cytotoxicity against the CLL-1^{dim} AML cells was limited *in vitro*^{20,21}. These results suggest the need for highly sensitive CARs that can react with CLL-1^{dim} AML cells to eradicate entire AML clones.

In this study, we developed CB-derived NK cells transduced with a CAR derived from the newly established monoclonal antibody 2-23, which has a high affinity for CLL-1. We compared the potential of 2-23 CAR NK cells with CAR NK cells derived from the widely used M26 mAb. Specifically, we examined the ability of 2-23 CAR NK cells and M26 CAR NK cells to eliminate AML cells with minimal CLL-1 expression *in vitro* and *in vivo*.

Materials and Methods

Ethical approval

The collection and use of human biospecimens were approved by the Institutional Review Boards of the participating institutions (IRB Protocol Nos. 18172-5 and 20472-5), and the study was conducted in accordance with the Declaration of Helsinki. Details are provided in the Supplementary Methods.

Generation of CAR NK cells

K562 cells expressing membrane-bound IL-15, IL-21, and 41BBL were generated by retroviral transduction and used as feeder cells²². Thirteen clones of mAbs against CLL-1 were generated as previously described²³. To generate a CAR construct, the isolated cDNAs of the variable regions of the κ light chain and heavy chain were fused with CD28 and CD3 ζ cDNAs by overlapping PCR. In addition, T2A-IL-15 cDNA was fused with the CAR. T-cell-depleted CB MNCs were stimulated with 100 Gy-irradiated feeder cells and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 20 IU/mL IL-2. After 1 week, retrovirus carrying the CAR-T2A-IL-15 cDNA was transduced into CB-derived NK cells using RetroNectin. The cells were then re-stimulated with 100 Gy-irradiated feeder cells, cultured for 1 or 2 additional weeks, and used for further experiments. As a control, CD19-targeting CAR NK cells were generated using an FMC63-derived scFv with CD28-CD3 ζ signaling domains. M26 CAR NK cells were generated using an scFv derived from the anti-CLL-1 monoclonal antibody M26, whose variable region sequences were obtained from the published patent (US Patent No. 10,723,805 B2). Similar to the 2-23 CAR construct, both the CD19 CAR and the M26 CAR included T2A-linked IL-15.

Cell lines

CLL-1-knockdown (KD) U937 cells were established by lentiviral infection of the

plasmid encoding for short-hairpin RNAs (Sigma-Aldrich), which target the CLL-1 mRNA for degradation. After transduction, cells were stained with anti-CLL-1-PE (Supplementary Table 2) and CLL-1^{dim} cells were purified by FACS for subsequent use.

Cytotoxicity assay

The ability of NK cells to lyse tumor target cells was measured using ⁵¹Cr release assays, as previously reported²⁴. For the IncuCyte cytotoxicity assay, U937 cells or a single-cell clone of CLL-1 knockdown (KD) U937 cells expressing luciferase-GFP were co-cultured with CAR NK cells (2-23 CAR NK, M26 CAR NK, or CD19 CAR NK) at an E:T ratio of 9:1 in RPMI 1640 supplemented with 10% FBS without IL-2. Live-cell imaging was performed using an IncuCyte S3 system (Sartorius) at 37°C with 5% CO₂.

In vivo xenograft mouse model

A disseminated AML model was established by intravenous injection of 5×10^5 HL60-luc, 5×10^4 wild-type U937-luc, or 5×10^4 U937-CLL-1^{dim}-luc cells (clone 2) into 6- to 9-week-old female NOG mice irradiated with 1.0 Gy. The next day, the mice were intravenously injected with 2-23 CAR NK cells, M26 CAR NK cells, or CD19 CAR NK cells (5×10^5 cells in the HL60-luc experiments and 2×10^6 cells in the wild-type U937-luc and U937-CLL-1^{dim}-luc experiments, respectively).

Intra-BM transplantation of BM cells from patients with AML was performed as

previously reported²⁴.

Statistical analysis

An unpaired two-tailed Student's t-test was used to determine statistically significant differences between samples. P-values < 0.05 were considered statistically significant.

Mouse survival data were analyzed using Kaplan–Meier survival curves, and a log-rank test was used to measure statistically significant differences.

Further details of the methods used are available in the Supplementary Appendix.

Results

CB-derived NK cells transduced with a CAR derived from the novel anti-CLL-1 monoclonal antibody (mAb) 2-23 exhibit a significant anti-leukemia effect

Thirteen novel mAbs that react with human CLL-1 were established by immunizing mice with BaF3 cells expressing CLL-1²³. A retroviral vector carrying a CAR derived from each anti-CLL-1 mAb was generated using the transmembrane/cytoplasmic regions of CD28 and the CD3ζ cytoplasmic region. IL-15 was co-expressed to enhance the proliferation of CAR NK cells *in vivo*, as reported previously²⁵ (Figure 1A). T-cell-depleted CB MNCs were co-cultured with irradiated K562-based feeder cells that expressed 4-1BB ligand, as well as membrane-bound IL-15 and IL-21²², in a medium

supplemented with IL-2. After seven days the cells were transduced with each CAR construct (Figure 1B). Among CAR NK cells derived from the 13 novel anti-CLL-1 mAbs (Supplementary Figure 1A), CAR NK cells derived from five anti-CLL-1 mAbs exhibited significant cytotoxicity against CLL-1-positive U937 AML cells *in vitro* (Supplementary Figure 1B). We selected CAR NK cells derived from the 2-23 mAb (termed 2-23 CAR NK cells) because we have recently reported that CAR T cells derived from the 2-23 mAb can exhibit an excellent anti-leukemia effect ²³. The 2-23 CAR NK cells could be efficiently established from CB cells obtained from various donors (Figure 1C, D; Supplementary Figure 2A, B) and exhibited cytotoxicity against AML cell lines (Figure 1E, F and G; Supplementary Figure 2C, D). Before culturing began, CD3-negative cells included B cells (CD19⁺), mature myeloid cells (CD14⁺), and hematopoietic progenitor cells (CD34⁺). Seven days after the start of culture, B cells, myeloid cells, or hematopoietic progenitor cells were barely detectable and never increased afterwards. (Supplementary Figure 3).

We further evaluated the *in vivo* anti-leukemic activity of 2-23 CAR NK cells using an HL60 xenograft model (Figure 2A). Treatment with 2-23 CAR NK cells markedly reduced leukemia burden (Figure 2B, C) and significantly prolonged survival compared with the control group (Figure 2D). No signs of toxicity, including decreased

activity, generalized malaise, piloerection, or weight loss, were observed in mice infused with the 2-23 or CD19 CAR NK cells (Figure 2E).

CB-derived NK cells transduced with a CAR derived from the 2-23 mAb, but not from M26, a widely used anti-CLL-1 mAb, could kill CLL-1^{dim} AML cells *in vitro* and enhance the survival of the mice transplanted with CLL-1^{dim} AML cells *in vivo*

The binding kinetics of 2-23 mAb to U937 AML cells were compared with those of M26 mAb, the source of the antigen-recognition domain of the anti-CLL-1 CAR in previous preclinical^{26,27} and clinical^{15,28} studies. The 2-23 mAb had a higher affinity for CLL-1 than the M26 mAb (Figure 3A). The EC50 was 0.0412 µg/mL (95% CI, 0.0304–0.0556) for 2-23 mAb and 2.408 µg/mL (95% CI, 2.196–2.651) for M26 mAb.

U937 cell clones that express minimal levels of CLL-1 (U937-CLL-1^{dim}) were established using shRNA transduction for CLL-1 and single-cell sorting with FACS (Figure 3B). CAR NK cells were developed using the 2-23 or M26 antigen-recognition regions (Figure 3C). Using IncuCyte live imaging system, we performed a cytotoxicity assay and found that the 2-23 CAR NK cells killed U937-CLL-1^{dim}, whereas the M26 CAR NK cells did not (Figure 3D).

Next, we evaluated the anti-leukemic activity of 2-23 CAR NK cells and M26

CAR NK cells *in vivo* using xenograft models (Figure 4A). Immunodeficient mice were transplanted with luciferase-expressing U937 CLL-1^{dim} AML cells. The following day, the mice were randomly divided into groups and injected intravenously with 2-23 CAR NK cells, M26 CAR NK cells, or CD19 CAR NK cells. Treatment with 2-23 CAR NK cells, but not with M26 CAR NK cells, reduced tumor burden and significantly prolonged survival compared to the control group (Figure 4B-D). In a xenograft model using the wild-type U937 AML cells, treatment with both 2-23 CAR NK cells and M26 CAR NK cells reduced leukemia burden, and no significant difference in survival was observed between two groups (Figure 4B-D).

CAR NK cells derived from 2-23 mAb expanded robustly, persisted, and eradicated AML cells in AML patient-derived xenograft models

The anti-leukemic effect of 2-23 CAR NK cells was also tested in patient-derived xenograft (PDX) models using two CLL-1-positive AML samples: AML #1, from a patient with AML who was experiencing early relapse after allo-HSCT, and AML #2, from a patient who was newly diagnosed as AML (Figure 5A, Supplementary Table 1). T-cell-depleted BM or PB cells from the two patients with AML were injected into the BM of 1-Gy-irradiated immunodeficient mice. Two weeks later, the mice were randomly

divided into two groups and received an intravenous injection of either 2-23 CAR NK cells or CD19 CAR NK cells. One month after the injection of CAR NK cells, the BM cells were analyzed by flow cytometry (Figure 5B). In mice treated with CD19 CAR NK cells, robust engraftment of human (h) CD45⁺hCLL-1⁺AML cells was detected (Figure 5C–F, Supplementary Figure 4A, B). In contrast, hCD45⁺ hCLL-1⁺ AML cells have disappeared completely in all mice treated with 2-23 CAR NK cells (Figure 5C–F, Supplementary Figure 4A, B). In addition, hCD45⁺hCD56⁺ CAR NK cells were robustly expanded in mice treated with 2-23 CAR NK cells, whereas the CAR NK cells were undetectable in mice treated with CD19 CAR NK cells (Figure 5C–F, Supplementary Figure 4A, B).

Discussion

In this study, we generated CAR NK cells derived from 13 new anti-CLL-1 mAbs. Among them, CAR NK cells derived from the 2-23 mAb were identified as having the potential to exert cytotoxicity against AML cells expressing CLL-1 *in vitro* and *in vivo*. The 2-23 mAb has a higher affinity for CLL-1 than the M26 mAb, which was used to construct the CAR evaluated in clinical trials^{15,28}. We demonstrated that 2-23 CAR NK cells react with CLL-1^{dim} leukemia cells that M26 CAR NK cells cannot recognize. Furthermore,

treatment with 2-23 CAR NK cells, but not M26 CAR NK cells, enhanced the survival of the mice transplanted with the U937-CLL-1^{dim} cells. Both 2-23 CAR NK cells and M26 CAR NK cells exhibited significant anti-leukemia effects against the wild-type U937 cells *in vitro* and *in vivo*. These results suggest that 2-23 CAR NK cells, but not M26 CAR NK cells, are sensitive enough to eliminate AML cells with minimal CLL-1 expression, whereas both 2-23 and M26 CAR NK cells are effective against AML cells with high CLL-1 expression. Therefore, 2-23 CAR NK cells may benefit many patients with AML by eradicating all AML clones. However, because CLL-1 is also expressed on normal myeloid cells, prolonged neutropenia is a potential concern associated with 2-23 CAR NK-cell therapy. As previously reported for CLL-1 CAR T-cell therapy¹⁷, 2-23 CAR NK-cell therapy may also be considered for use as a bridge to allo-HSCT rather than as a stand-alone therapy.

Previous studies have reported that NK cells do not expand or persist *in vivo* as efficiently as T cells²⁹. Consistent with these findings, the control NK cells, which expressed IL-15 but did not receive CAR signals, disappeared completely one month after infusion in our experiments. However, CAR signals have the potential to enable NK cells to expand and persist *in vivo*. In this study and in our recent report on CB-derived CAR NK cells that target a different molecule²⁴, robust expansion and long

persistence *in vivo* were observed in the CAR NK cells but not in the control NK cells.

Furthermore, administrating 2-23 CAR NK cells eradicated patient-derived AML cells

that had been engrafted in immunodeficient mice. These results confirm the high

potential of CB-derived CAR NK cells to expand and persist *in vivo*, as demonstrated in

clinical trials of CB-derived CD19 CAR NK cells^{2,3}, whereas the possibility of

immunological rejection by the host immune system should be considered for

allogeneic CAR NK cells.

In summary, our findings suggest that CB-derived 2-23 CAR NK cells can

eliminate AML cells with minimal CLL-1 expression. Therefore, CB-derived 2-23 CAR

NK cells have the potential to benefit patients who are insensitive to chemotherapy and

undergo allo-HSCT while still in the non-remission stage, as they can eradicate entire

AML clones before allo-HSCT.

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Figure 1. CB-derived NK cells transduced with a CAR derived from the novel anti-CLL-1 monoclonal antibody (mAb) 2-23 exhibit a significant anti-leukemia effect in vitro.

(A) CAR construct derived from anti-human CLL-1 mAbs. (B) Scheme showing the CAR NK cell generation protocol. (C) Flow cytometry analysis of CD56, CD3, and CAR expression on the 2-23 CAR NK cells or CD19 CAR NK cells. (D) The number of CD3⁺CD56⁺ NK cells derived from 1×10^5 CB MNCs at the indicated time points during 2-23 CAR NK or CD19 CAR NK cell production. (E) ^{51}Cr release assay for the measurement of the specific lysis of CLL-1-expressing target cells (HL60 or U937) by 2-23 CAR NK cells or CD19 CAR NK cells. Representative results obtained from a single CB unit (CB-1) are shown in C–E. Results from other CB units are shown in Supplementary Figure 2. (F) HL60 or U937 target cells were co-cultured with 2-23 CAR NK cells or CD19 CAR NK cells for 48 hours and analyzed by flow cytometry. (G) Absolute numbers of residual target cells after 48 hours of co-culture. In (D), (E) and (G), each experiment was performed in triplicate, and the mean $\pm$ standard deviation is shown.

Figure 2. 2-23 CAR NK cells exhibit a significant anti-leukemia effect in vivo.

(A) Experimental design for B–E. (B) Bioluminescence imaging of mice infused with either the 2-23 CAR NK cells or CD19 CAR NK cells (5×10^5 total NK cells per mouse,

n = 5 per group). (C) Quantification of whole-body luminescence (average radiance) of each mouse. In the 2-23 CAR NK group, the curves of two mice overlap along the x-axis. (D) Survival curves of the mice infused with either 2-23 CAR NK cells or CD19 CAR NK cells. P-value was determined by the log-rank test. (E) Body weight changes in mice treated with 2-23 CAR NK cells or CD19 CAR NK cells after tumor injection.

Figure 3. CB-derived NK cells transduced with a CAR derived from the 2-23 mAb, but not from M26, a widely used anti-CLL-1 mAb, could kill CLL-1^{dim} AML cells *in vitro*.

(A) Binding kinetics of the anti-CLL-1 mAbs 2-23 and M26 to U937 cells. The mean fluorescence intensity (MFI) values of 2-23 and M26 binding to U937 cells at various mAb concentrations are plotted. (B) Flow cytometric analysis of CLL-1 expression in the wild-type U937 cells and the CLL-1 knockdown clones (U937-CLL-1^{dim} clones 1 and 2). (C) Flow cytometric analysis of CD56, CD3, and CAR expression in 2-23 CAR NK cells, M26 CAR NK cells, and CD19 CAR NK cells. (D) IncuCyte live-cell imaging assay analyzing the killing of the wild-type U937 and the CLL-1 knockdown clones (U937-CLL-1^{dim} clones 1 and 2) by 2-23 CAR NK cells, M26 CAR NK cells, or CD19 CAR NK cells. Tumor cell growth was monitored over the indicated time and normalized to the value at time 0. This experiment was performed in triplicate, and the mean $\pm$ standard

deviation is shown.

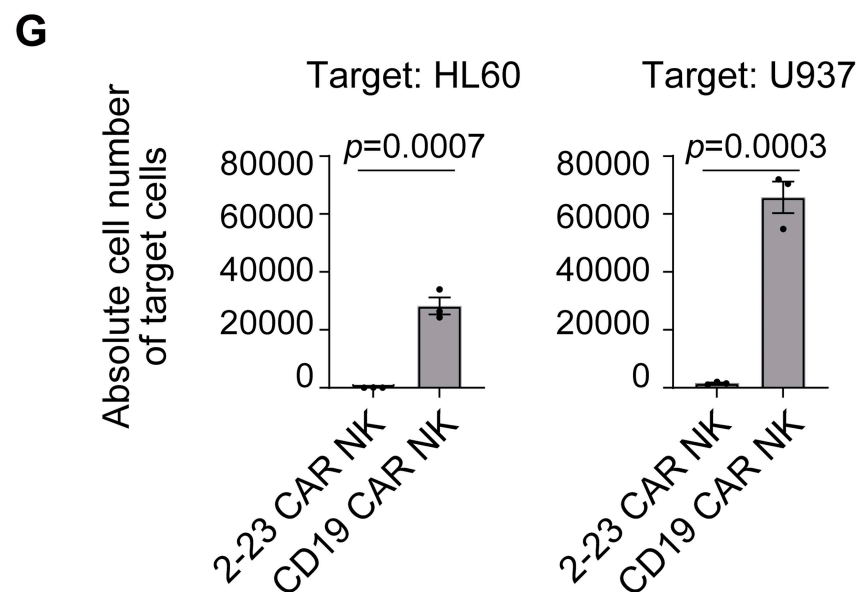
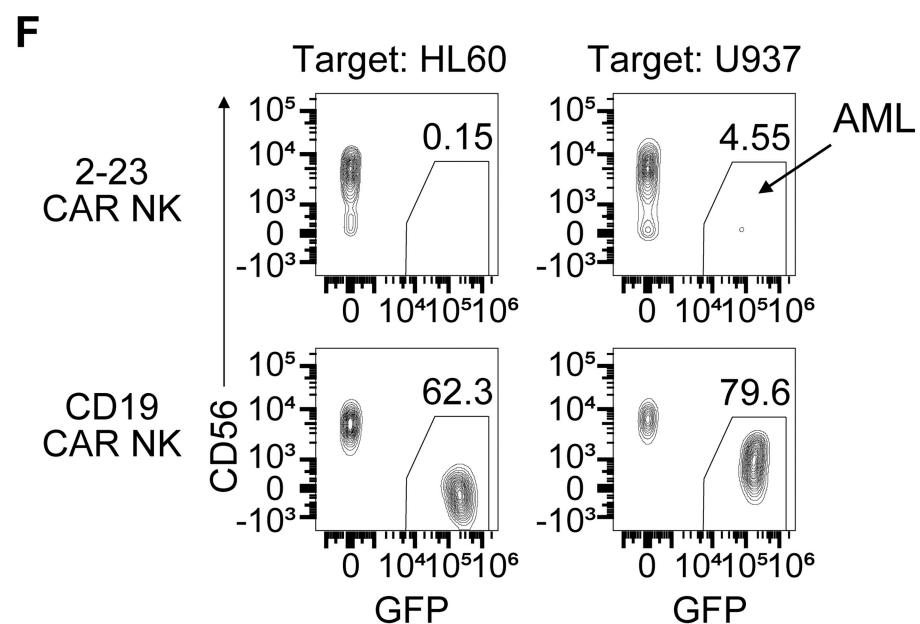
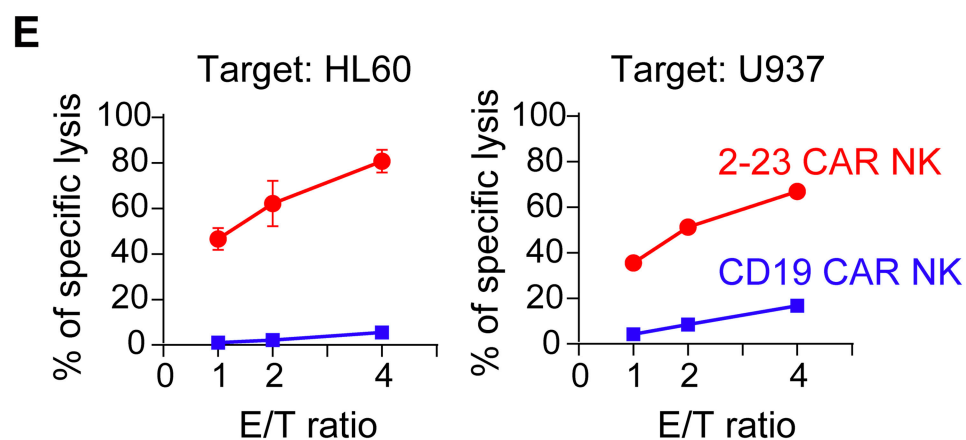
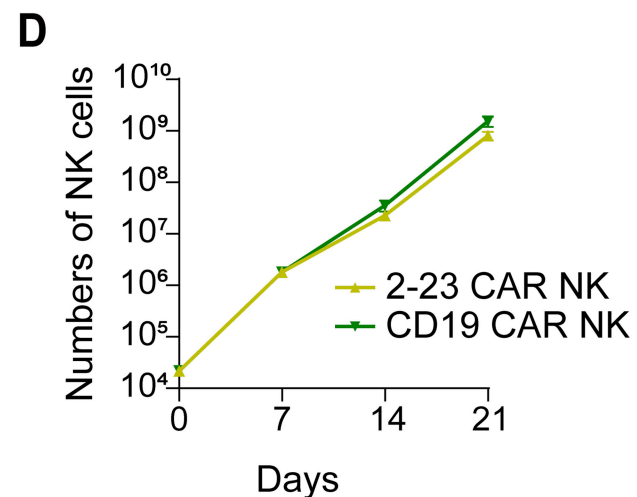
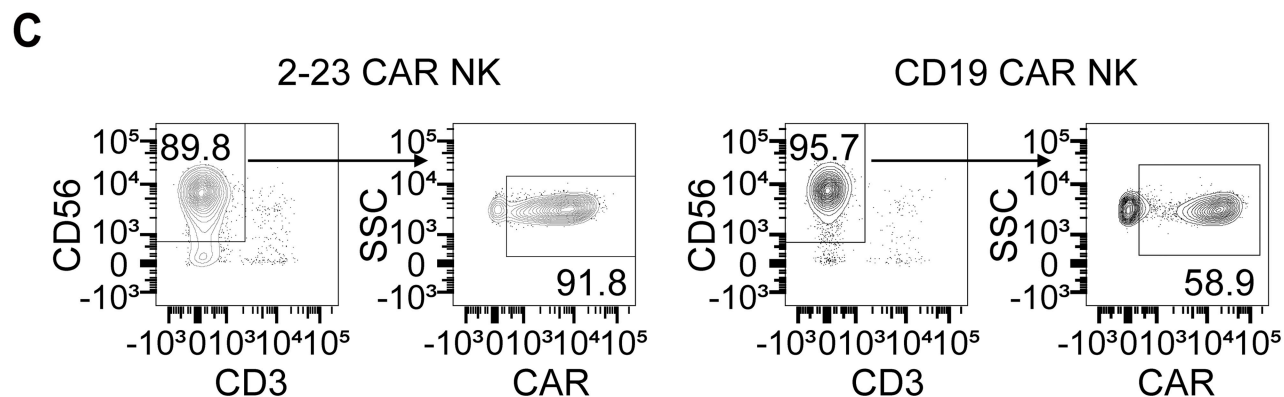
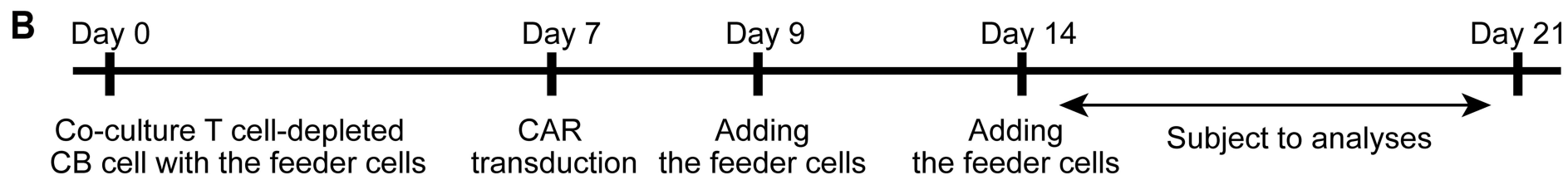
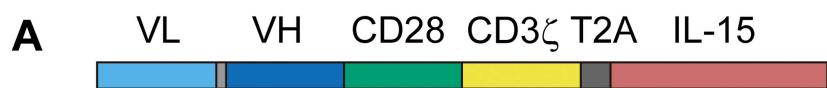
Figure 4. 2-23 CAR NK cells prolonged survival in mice transplanted with CLL-1^{dim} AML cells *in vivo*.

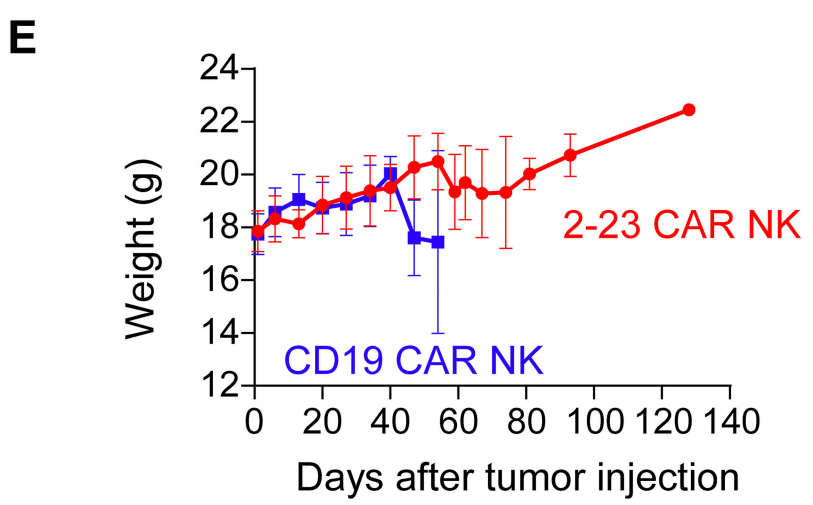
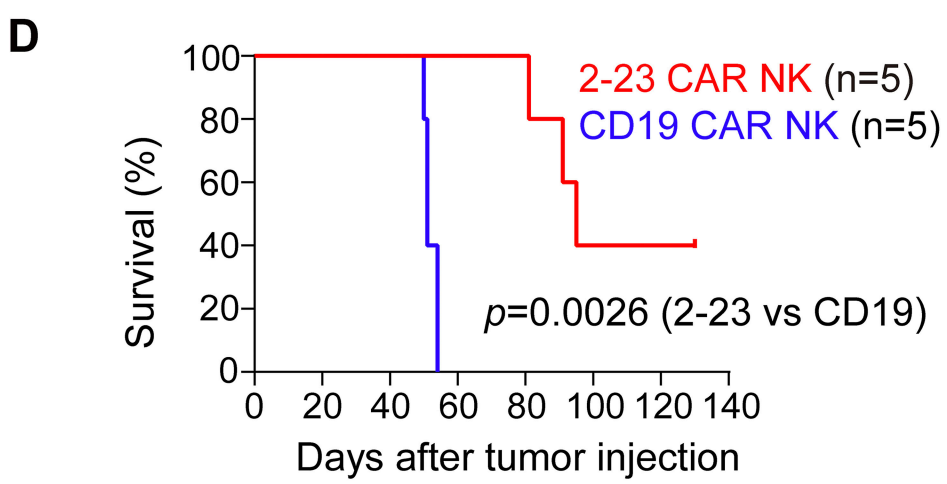
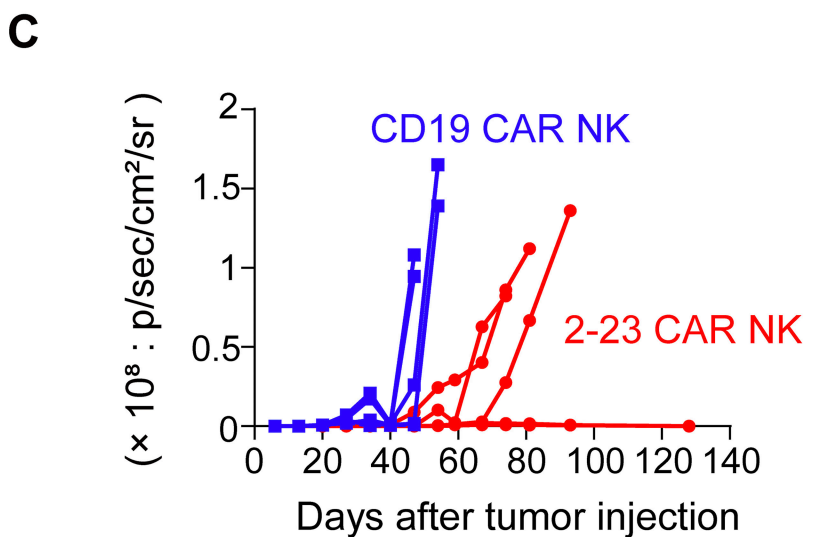
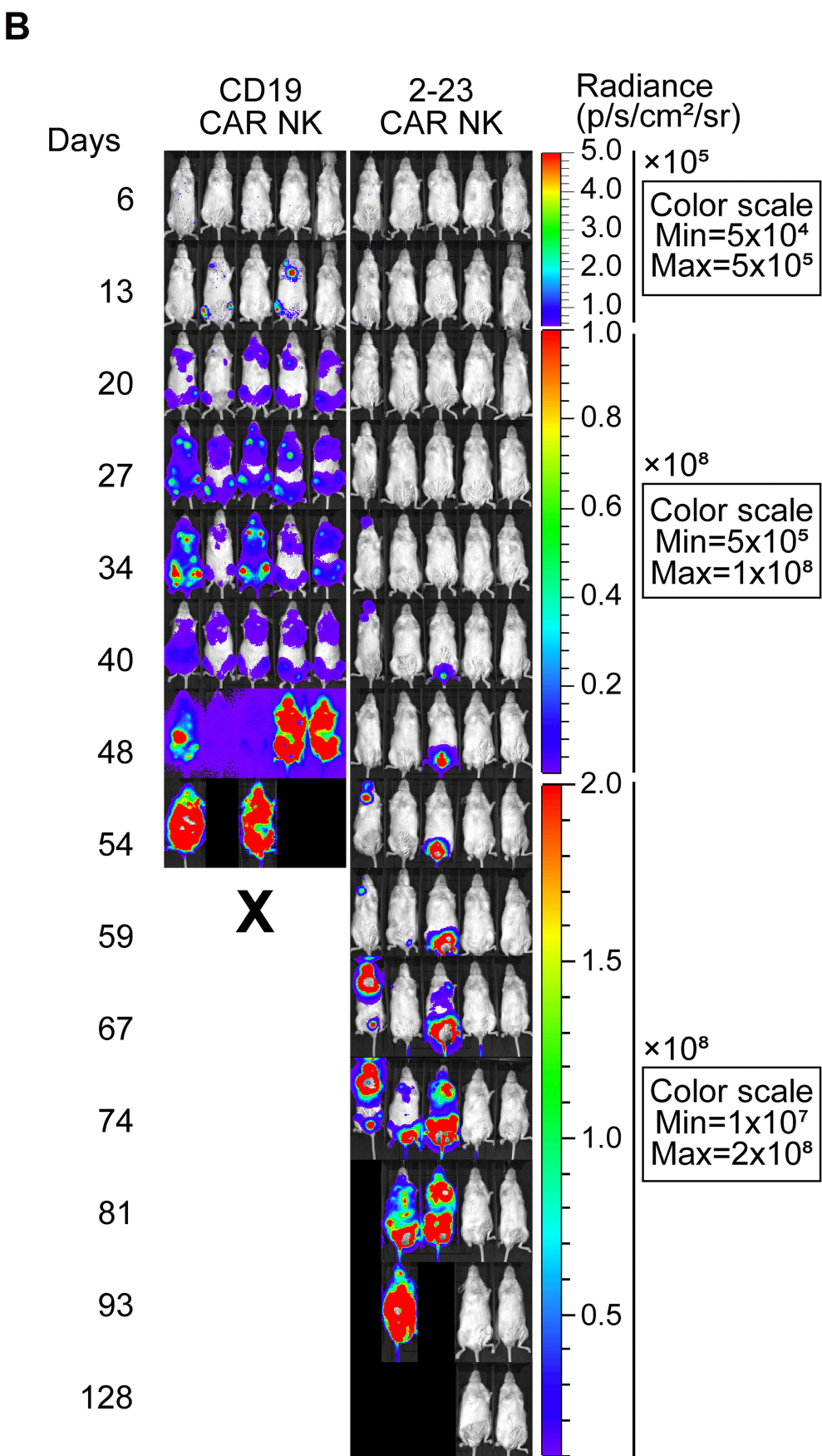
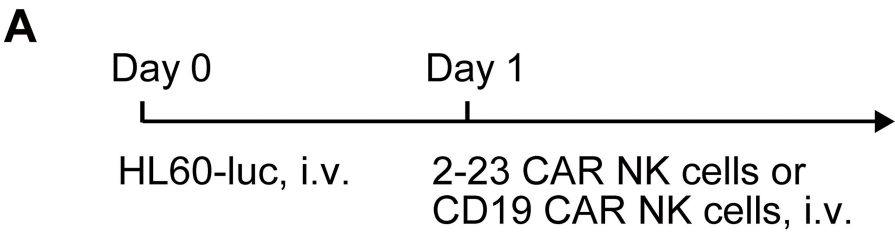
(A) Experimental design. The left panels show the results using U937-CLL-1^{dim} cells (clone 2). The right panels show the results using wild-type U937 cells. (B) Bioluminescence imaging of mice infused with 2-23 CAR NK cells, M26 CAR NK cells, or CD19 CAR NK cells (2×10^6 total NK cells per mouse). (C) Quantification of whole-body luminescence (average radiance) of each mouse. (D) Survival curves of mice infused with 2-23 CAR NK cells, M26 CAR NK cells, or CD19 CAR NK cells. P-values were determined by the log-rank test.

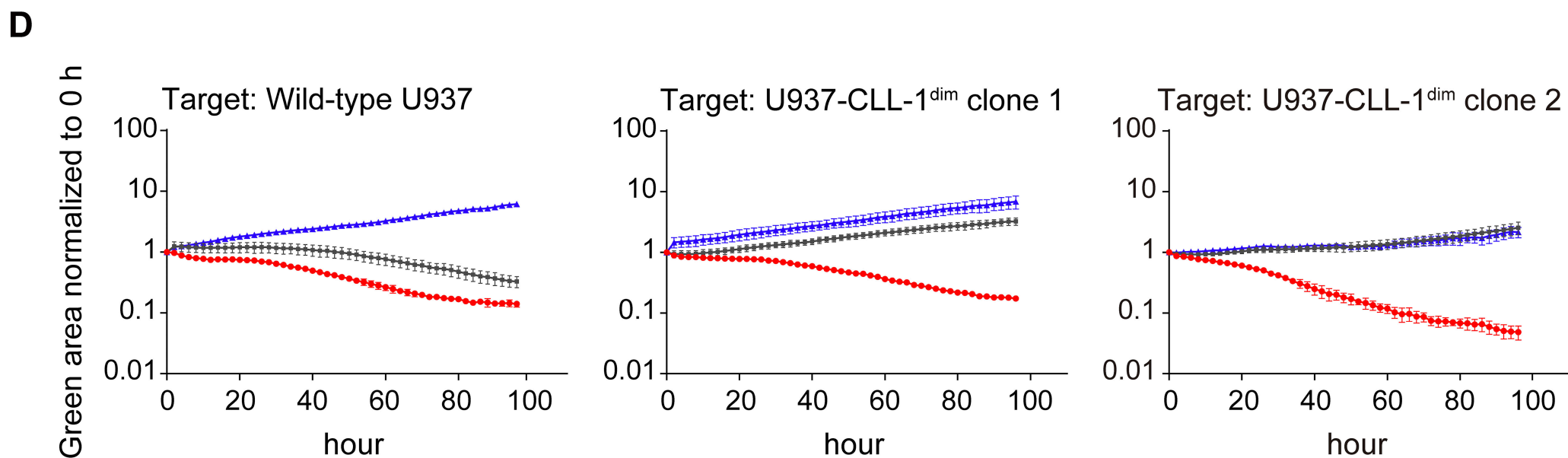
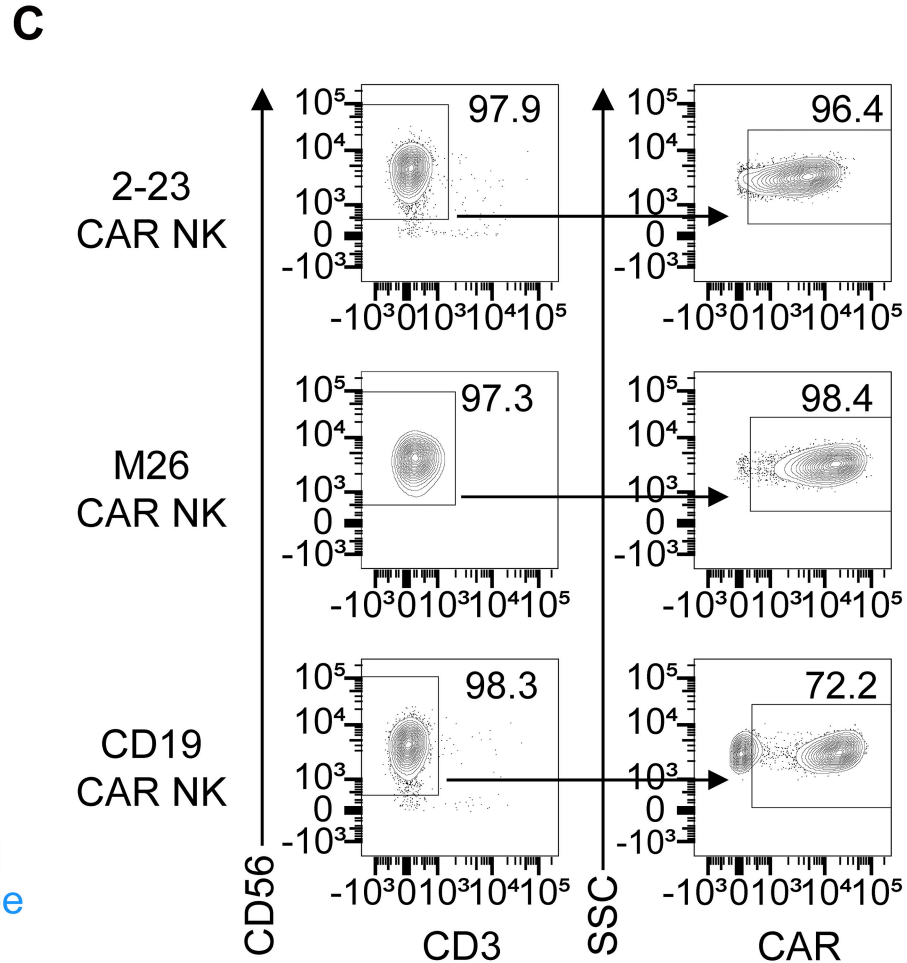
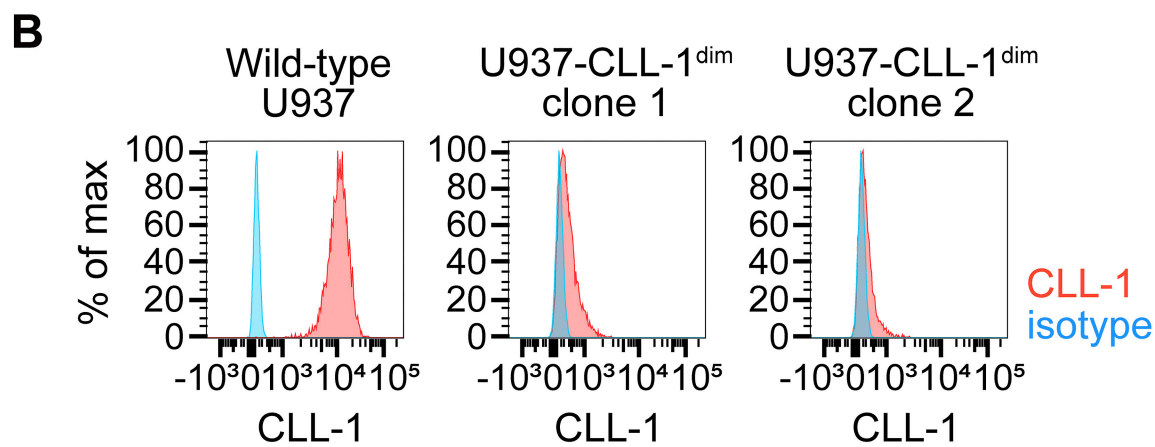
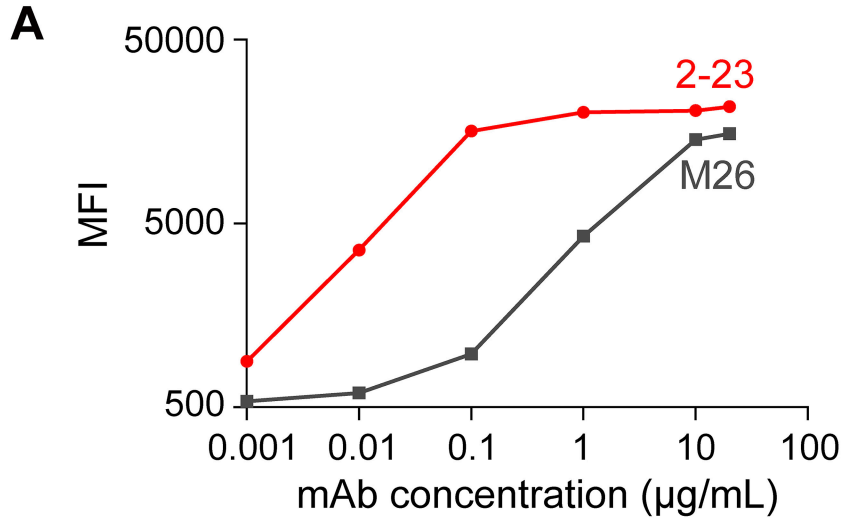
Figure 5. 2-23 CAR NK cells expanded robustly, persisted, and eradicated AML cells in AML patient-derived xenograft models.

(A) Flow cytometric analysis of CLL-1 expression on AML cells from the BM of AML #1 and the PB of AML #2. (B), Experimental design for C–F. (C), (D), Patient-derived xenograft (PDX) models were established using AML cells from AML #1 (C, n = 4 per group) and AML #2 (D, n = 3 per group). BM cells were analyzed by flow cytometry 28 days (AML #1) or 34 days (AML #2) after infusion of either 2-23 CAR NK cells or CD19

CAR NK cells. Plots were pre-gated on live mTer119⁻ cells. (E), (F), Quantification of the percentages of hCD45⁺hCLL-1⁺ AML cells (left) and hCD45⁺hCD56⁺CAR⁺ (CAR NK) cells (right) among mCD45⁻hCD45⁺ BM cells. Data are presented as the mean (SD). Error bars indicate standard deviation. P-values in (E) and (F) were calculated using two-tailed Student's t-test.



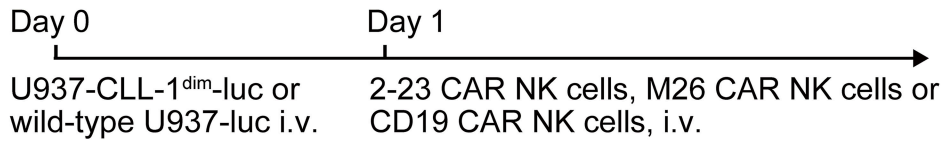
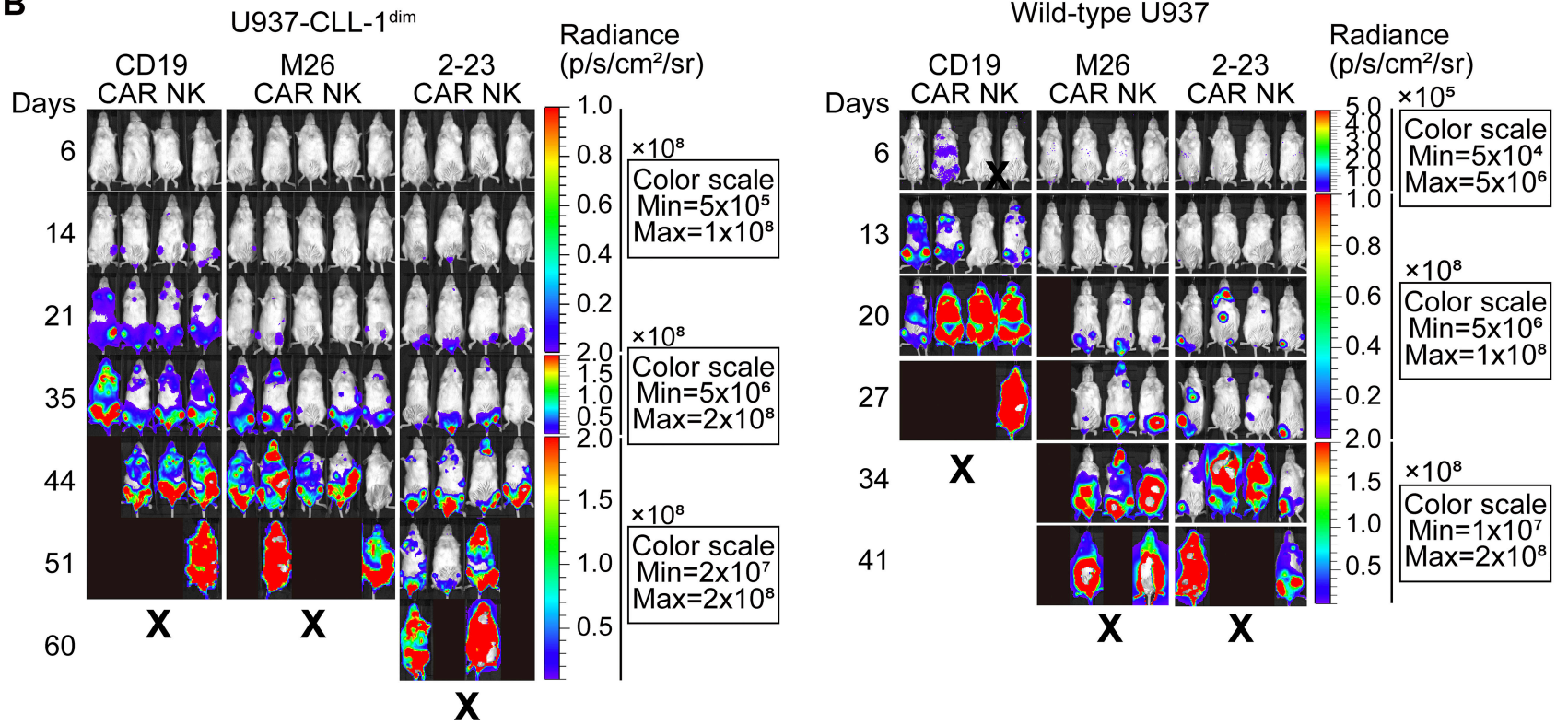
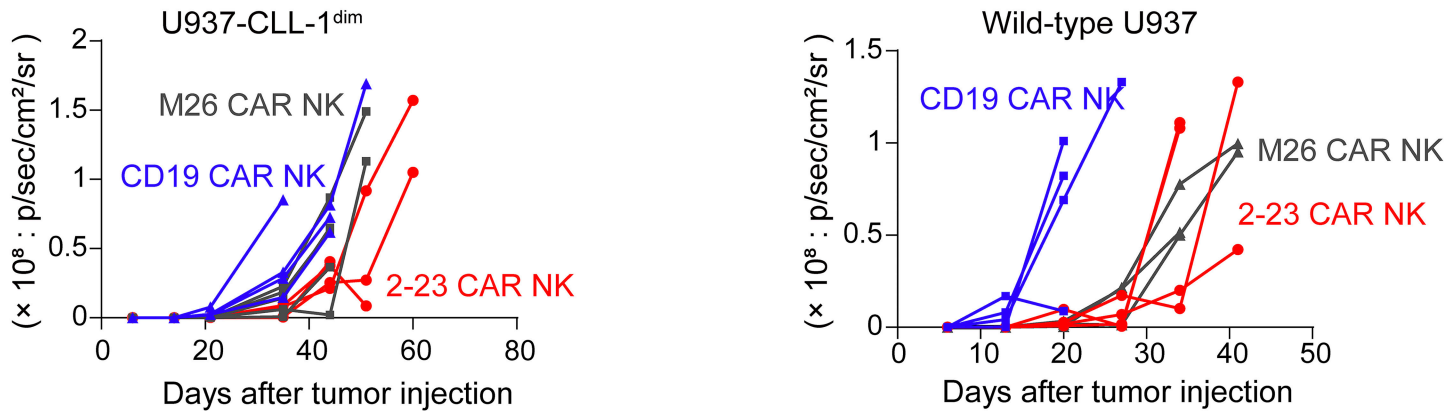
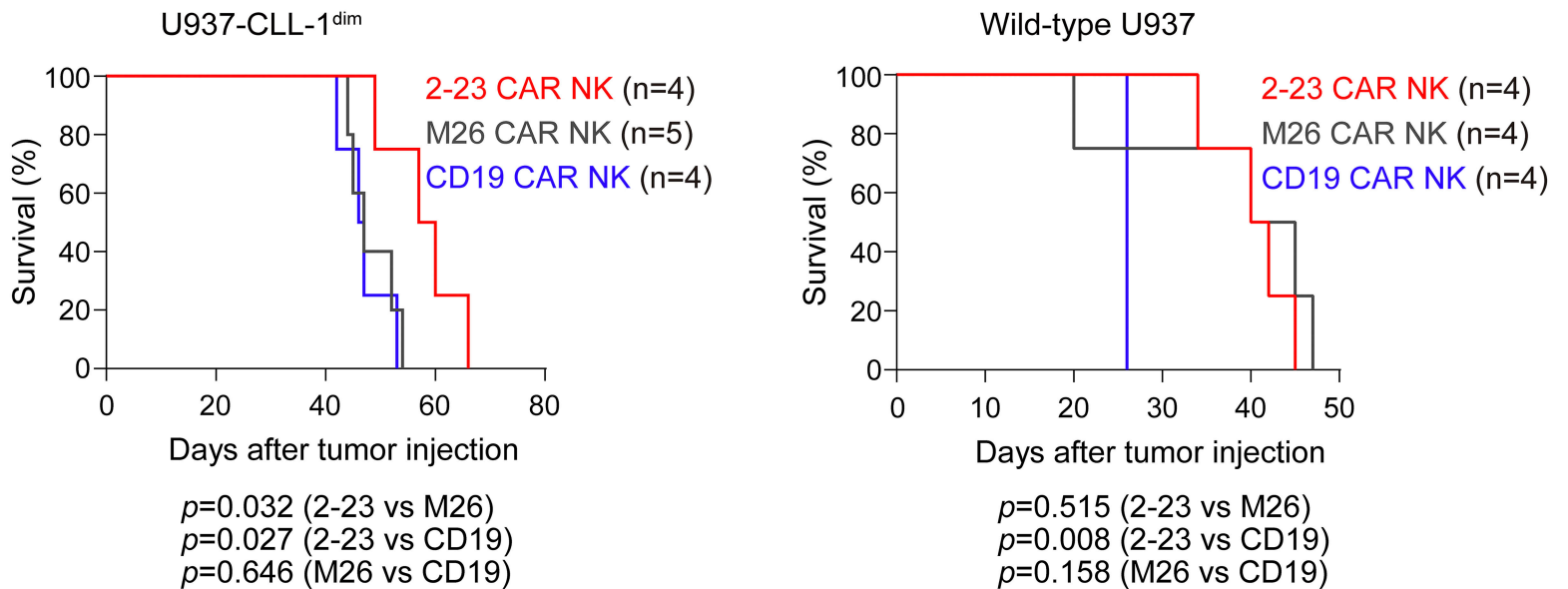




—●— 2-23 CAR NK

—■— M26 CAR NK

—▲— CD19 CAR NK

A**B****C****D**

Supplementary Appendix

Cord blood-derived natural killer cells engineered with a novel, high-affinity chimeric antigen receptor can eliminate acute myeloid leukemia cells with minimal C-type lectin-like molecule-1 expression

Materials and Methods

Ethical approval

All mouse experiments were approved by the administrative panel on laboratory animal care at the University of Osaka (Protocol No. 03-045-023). Human biospecimen collection and use were approved by the Institutional Review Boards of the University of Osaka Hospital, Osaka Metropolitan University Graduate School of Medicine, Osaka City General Hospital, Itami City Hospital, Hyogo Prefectural Nishinomiya Hospital, Hyogo Medical University, Suita Municipal Hospital, Osaka International Cancer Institute, Osaka National Hospital, Ashiya Municipal Hospital, Toyonaka Municipal Hospital, Sumitomo Hospital, Nippon Life Hospital, Minoo Municipal Hospital, and Osaka General Hospital of West Japan Railway Company (Institutional Review Board [IRB] Protocol No. 18172-5). The use of human CB for research purposes was approved

by the Institutional Review Boards of the University of Osaka Hospital, Kinki Cord Blood Bank, and Hyogo Cord Blood Bank (IRB Protocol No. 20472-5).

Patient samples and CB

Peripheral blood (PB) or bone marrow (BM) cells were collected from patients with AML after obtaining informed consent. Mononuclear cells (MNCs) were purified using Ficoll-Paque (GE Healthcare) and analyzed. CB cells were obtained from the Hyogo Cord Blood Bank and the Kinki Cord Blood Bank after obtaining informed consent.

Cell lines

The cell lines used in this study included 293T cells (CRL-3216), purchased from the American Type Culture Collection; K562 (JCRB0019), U937 (JCRB9021), and HL60 (JCRB0085) cells, purchased from the Japanese Collection of Research Bioresources Cell Bank; and U937-luc/GFP and HL60-luc/GFP cells, which were established by retroviral transduction of luciferase-IRES-GFP and fluorescence-activated cell sorting (FACS) of GFP^{high} cells. CLL-1-knockdown (KD) U937 cells were established by lentiviral infection of the plasmid encoding for short-hairpin RNAs (Sigma-Aldrich), which target the CLL-

1 mRNA for degradation (shRNA: CCGGCGCACCTGACTTAAATAACATCTCGAGATGTTATTTAAGTCAGGTGCGT TTTTG). After transduction, cells were stained with anti-CLL-1-PE and CLL-1^{dim} cells

were purified by FACS for subsequent use. U937, HL60, and feeder cells (K562 cells engineered to express 4-1BBL, mbIL-15 and mbIL-21) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). HEK293T cells used for lipofection were cultured in DMEM supplemented with 10% FBS.

Flow cytometry

PB, BM, and CB cells were stained with fluorochrome-conjugated mAbs after nonspecific binding was blocked by incubation with Human Serum AB (Gemini Bio) and FcR blocking reagent (Miltenyi Biotech). The antibodies used in this study are listed in Supplementary Table 2. Flow cytometry and cell sorting were performed using BD FACS Canto II, FACS Celesta, FACS Aria II, or FACS Aria Fusion (all from BD Biosciences) with BD FACSDIVA software (BD Biosciences) and analyzed with FlowJo software (BD Biosciences). In the analysis of BM and PB cells from patients with AML, AML cells were identified based on the expression of CD34 and CLL-1.

Generation of CAR NK cells

K562 cells expressing membrane-bound IL-15, IL-21, and 41BBL were generated by retroviral transduction and used as feeder cells¹. Thirteen clones of mAbs against CLL-1 were generated by immunizing mice with CLL-1-transduced mouse BaF3 cells and generating hybridoma cells². The cDNA of the variable region of each mAb was obtained

from hybridoma cells by 5' Rapid Amplification of cDNA ends (RACE) PCR with a Smarter RACE PCR kit (Takara Bio) and then sequenced. To generate a CAR construct, the isolated cDNAs of the variable regions of the κ light chain and heavy chain were fused with CD28 and CD3 ζ cDNAs by overlapping PCR. In addition, T2A-IL-15 cDNA was fused with the CAR. T cells were removed using CD3 MicroBeads (Miltenyi Biotec).

The linker sequence used for the scFv was GGCTCCACTAGCGGTTCCGGCAAACCTGGCAGCGGAGAAGGCAGC. The hinge and transmembrane (TM) domains were derived from human CD28. The hinge domain was

ATTGAAGTTATGTATCCTCCTCCTTACCTAGACAATGAGAAGAGCAATGGAAC
CATTATCCATGTGAAAGGGAAACACCTTTGTCCAAGTCCCCTATTTCCCGGAC
CTTCTAAGCCC, and the transmembrane (TM) domain was
TTTTGGGTGCTGGTGGTGGTTGGTGGAGTCCTGGCTTGCTATAGCTTGCTAGT
AACAGTGGCCTTTATTATTTCTGGGTG. T-cell-depleted CB MNCs were stimulated with 100 Gy-irradiated K562-mbIL-15-mbIL-21-41BBL cells and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 20 IU/mL IL-2. After 1 week, retrovirus carrying the CAR-T2A-IL-15 cDNA was transduced into CB-derived NK cells using RetroNectin. The cells were then re-stimulated with 100 Gy-irradiated

K562-mbIL-15-mbIL-21-41BBL cells, cultured for 1 or 2 additional weeks, and used for further experiments. CB units were preliminarily tested for proliferation after stimulation with K562 feeder cells and IL-2, and those with high proliferative potential were used for the experiments. As a control, CD19-targeting CAR NK cells were generated using an FMC63-derived scFv with CD28–CD3 ζ signaling domains. M26 CAR NK cells were generated using an scFv derived from the anti-CLL-1 monoclonal antibody M26, whose variable region sequences were obtained from the published patent (US Patent No. 10,723,805 B2). Similar to the 2-23 CAR construct, both the CD19 CAR and the M26 CAR included T2A-linked IL-15.

Cytotoxicity assay

The ability of NK cells to lyse tumor target cells was measured using ^{51}Cr release assays, as previously reported³. Alternatively, CAR NK cells were co-cultured with GFP-expressing HL60 or U937 cells at an E/T ratio of 4:1 for 48 h, and the surviving leukemia cells were measured by flow cytometry. The absolute number of surviving leukemia cells was calculated by multiplying the number of viable cells by the percentage of GFP-positive cells.

For the IncuCyte cytotoxicity assay, U937 cells or a single-cell clone of CLL-1 knockdown (KD) U937 cells expressing luciferase–GFP were seeded at 1×10^4 cells per

well in 96-well flat-bottom plates in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) without IL-2. CAR NK cells (2-23 CAR NK, M26 CAR NK, or CD19 CAR NK) were then added at 9×10^4 cells per well, corresponding to E/T ratio of 9:1. Each condition was tested in technical triplicate wells. Live-cell imaging was performed using an IncuCyte S3 system (Sartorius) at 37°C with 5% CO₂. Images were acquired every 2 h from four distinct fields per well using the green fluorescence channel. GFP-positive area was quantified for each field, and the mean value per well was calculated. Data were normalized to the GFP-positive area at time 0, immediately prior to CAR NK cell addition.

In vivo xenograft mouse model

A disseminated AML model was established by intravenous injection of 5×10^5 HL60-luc, 5×10^4 wild-type U937-luc, or 5×10^4 U937-CLL-1^{dim}-luc cells (clone 2) into 6- to 9-week-old female NOG mice irradiated with 1.0 Gy. The next day, the mice were intravenously injected with 2-23 CAR NK cells, M26 CAR NK cells, or CD19 CAR NK cells (5×10^5 cells in the HL60-luc experiments and 2×10^6 cells in the wild-type U937-luc and U937-CLL-1^{dim}-luc experiments, respectively). The mice were analyzed using In Vivo Imaging System (IVIS) at the indicated time points.

Primary AML samples were collected from patients' bone marrow or peripheral blood. Mononuclear cells were isolated by Ficoll density gradient centrifugation and

cryopreserved until use. After thawing, the cells were resuspended in RPMI 1640 (Nacalai Tesque) supplemented with 10% FBS (Nichirei) and 80 U/mL DNase I (Sigma-Aldrich). CD3⁺ cells were depleted using CD3 MicroBeads (Miltenyi Biotec), and the remaining cells were immediately transplanted into immunodeficient mice via intra-bone marrow transplantation (iBMT) without prior *in vitro* culture. Intra-BM transplantation of BM cells from patients with AML was performed as previously reported³. Six- to eight-week-old female NOG mice were irradiated with 1.0 Gy 4–24 h before transplantation, then injected in the left tibia with 4×10^5 CD3-depleted AML cells. Thirteen days later, 2×10^6 2-23 CAR NK cells or CD19 CAR NK cells were administered intravenously. One month after infusion, the BM cells were analyzed by flow cytometry.

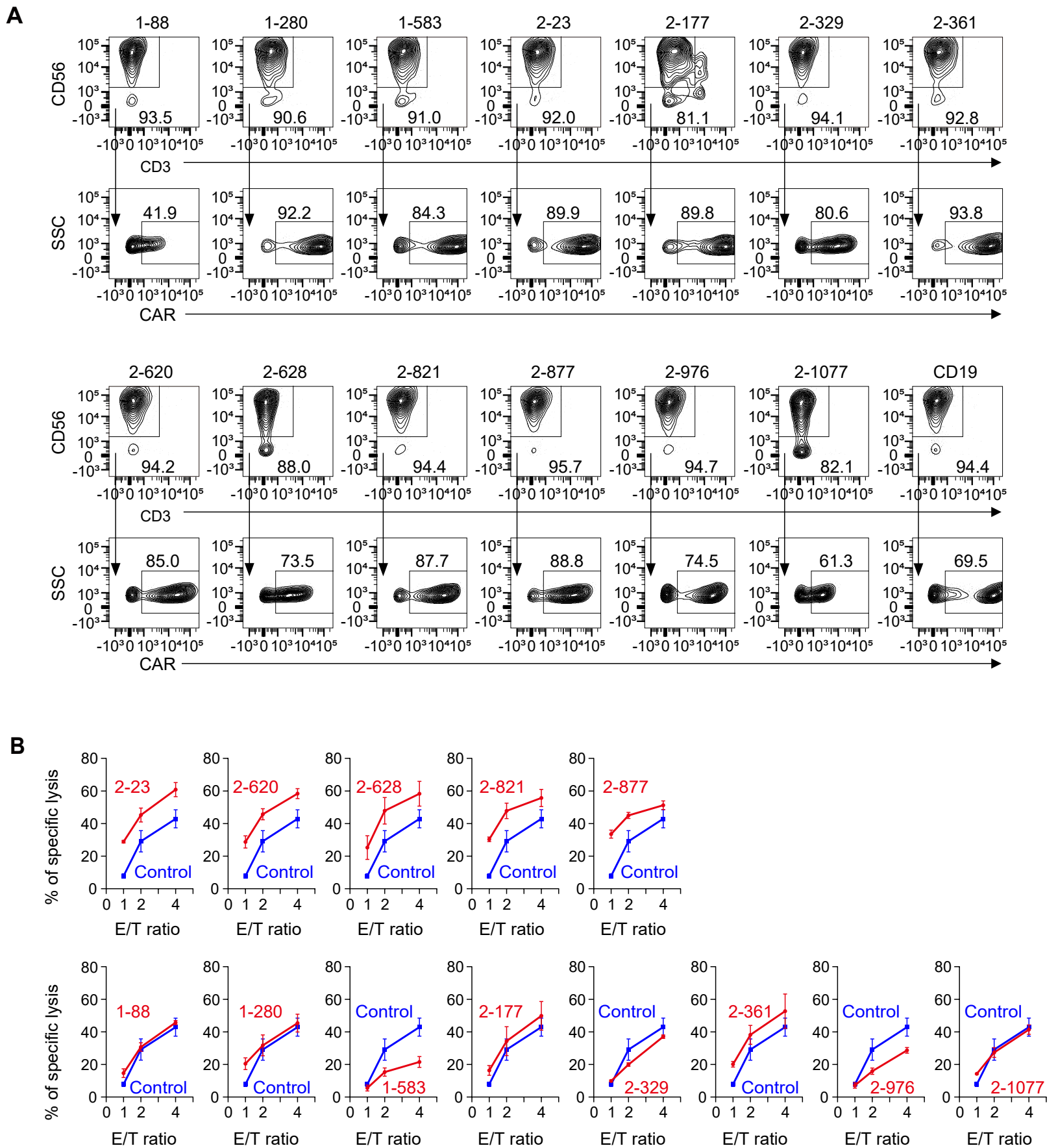
Mice were euthanized by cervical dislocation under isoflurane anesthesia when moribund or per humane endpoint criteria. All procedures conformed to the NIH guidelines and ARRIVE reporting standards.

Statistical analysis

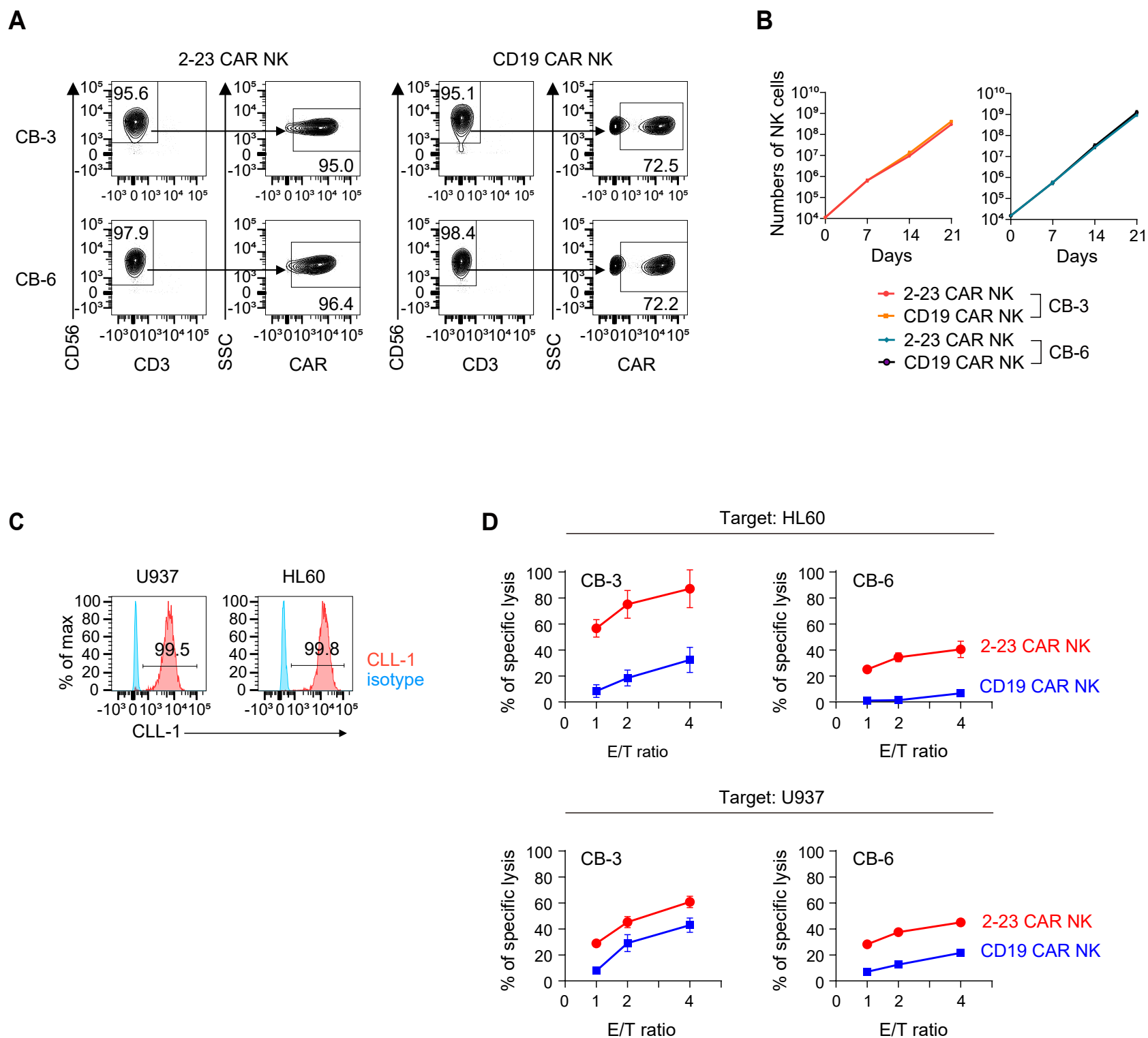
An unpaired two-tailed Student's t-test was used to determine statistically significant differences between samples. P-values < 0.05 were considered statistically significant. Mouse survival data were analyzed using Kaplan–Meier survival curves, and a log-rank test was used to measure statistically significant differences. JMP Pro 15 or GraphPad

Prism 10 (GraphPad Software) was used for statistical analyses. For the animal studies, no statistical methods were used to pre-determine the sample sizes; however, the sample sizes were similar to those reported in previous publications^{1,3}. Data collection and analysis were not performed in a manner that was blind to the experimental conditions.

1. Kogue Y, Ikeda S, Maehara Y, et al. CAR NK cell production from human cord blood NK progenitor cells is enhanced by stimulation of the IL-15 receptor. *Cancer Immunol Immunother.* 2025;74(11):338.
2. Kida S, Suga M, Yamaguchi Y, et al. CAR T cells derived from a novel, high-affinity anti-CLL-1 monoclonal antibody exhibit a significant anti-AML effect. *Cancer Immunol Immunother.* 2026;75(3).
3. Ikeda S, Hasegawa K, Kogue Y, et al. CAR T or NK cells targeting mismatched HLA-DR molecules in acute myeloid leukemia after allogeneic hematopoietic stem cell transplant. *Nat Cancer.* 2025.



Supplementary Figure 1. Generation and cytotoxicity of CAR NK cells derived from 13 novel anti-CLL-1 monoclonal antibodies. **A.** Flow cytometry analysis of CD56, CD3, and CAR expression in CAR NK cells derived from the 13 novel anti-CLL1 monoclonal antibodies (mAbs). **B.** ^{51}Cr release assay measuring the specific lysis of U937 cells by CAR NK cells derived from the 13 novel anti-CLL1 mAbs (red lines) or CD19 CAR NK cells (blue lines). Five of the 13 CAR NK cells tested (2-23, 2-620, 2-628, 2-821, and 2-877) demonstrated specific lysis of U937 cells. Data are shown as mean of triplicate wells. Error bars indicate standard deviation.



Supplementary Figure 2. CB-derived NK cells transduced with a CAR derived from the novel anti-CLL-1 monoclonal antibody (mAb) 2-23 exhibit a significant anti-leukemia effect *in vitro*. **A**, Flow cytometry analysis of CD56, CD3, and CAR expression on the 2-23 CAR NK cells or CD19 CAR NK cells. **B**, The number of CD3-CD56⁺ NK cells derived from 1×10^5 CB MNCs at the indicated time points during 2-23 CAR NK or CD19 CAR NK cell production. **C**, Flow cytometry analysis of CLL-1 expression in U937 and HL60 cells. **D**, ^{51}Cr release assay for the measurement of the specific lysis of CLL-1-expressing target cells (HL60 or U937) by 2-23 CAR NK cells or CD19 CAR NK cells. In **(B)** and **(D)**, each experiment was performed in triplicate, and the mean $\pm$ standard deviation is shown.



Supplementary Figure 3. Lineage composition of NK cell cultures during *in vitro* expansion. Flow cytometric analysis of CD56, CD3, CD16, CD14, CD19, and CD34 expression in the 2-23 CAR NK cells. Cells derived from three independent cord blood units (CB-1, CB-3, and CB-6) were analyzed at days 0, 7, 13, and 21 of culture.

Supplementary Table 1. Patient characteristics of the patient-derived xenograft (PDX) model

UPN	Age, Sex	Sample type	Disease status	Gene mutation	Karyotype and cytogenetics
AML #1	44, F	BM	de novo AML, relapsed after allo-HSCT	FLT3-ITD, TP53, NPM1	45, XX, dic(12;17)(p11.2;p11.2) 45, idem, -2, add(4)(q21), add(18)(q21), +mar1
AML #2	60, F	PB	de novo AML, newly diagnosed	FLT3-ITD	48XX, add(1)(p11), add(2)(p11.2), +5,del(5)(q)×2, +8

Supplementary Table 2. List of antibodies for flow cytometry

Name	Fluorochrome	Reactivity	Cat#	Clone	Source	Dilution
CD3	Cy7PE	Human	300420	UCHT1	BioLegend	1:200
CD3	Alexa Fluor 700	Human	300424	UCHT1	BioLegend	1:100
CD14	BV510	Human	301841	M5E2	BioLegend	1:200
CD19	FITC	Human	302206	HIB19	BioLegend	1:50
CD34	APC	Human	340441	8G12	BD Biosciences	1:100
CD34	BV421	Human	343610	561	BioLegend	1:400
CD45	Cy7APC	Human	368516	2D1	BioLegend	1:50
CD56	PE	Human	318306	HCD56	BioLegend	1:1600
CD56	FITC	Human	318303	HCD56	BioLegend	1:50
CLL-1	PE	Human	353604	50C1	BioLegend	1:800
CLL-1	APC	Human	353606	50C1	BioLegend	1:200
TIM3	Cy7PE	Human	345013	F38-2E2	BioLegend	1:50
TIGIT	BV421	Human	372710	A15153G	BioLegend	1:50
CD16	BV510	Human	302048	3G8	BioLegend	1:50
CD16	APCCy7	Human	302017	3G8	BioLegend	1:50
PD-1	BV605	Human	563245	EH12.1	BD Biosciences	1:50
LAG3	BV785	Human	744727	T47-530	BD Biosciences	1:50
NKp80	APC	Human	346708	5D12	BioLegend	1:50
NKG2D	Cy7PE	Human	320812	1D11	BioLegend	1:50
NKG2A	Cy7PE	Human	375114	S19004C	BioLegend	1:50
NKp46	Cy7PE	Human	331916	9E2	BioLegend	1:50
NKp30	Cy7PE	Human	325230	P30-15	BioLegend	1:50
CD45	Cy7PE	Mouse	103114	30-F11	BioLegend	1:3200
Ter119	PerCPCy5.5	Mouse	116228	Ter-119	BioLegend	1:50
F(ab') ₂	Alexa Fluor 647	Mouse	115-605-072	polyclonal	Jackson ImmunoResearch	1:100
IgG	PE	Mouse	405307	polyclonal	BioLegend	1:200
CD19CAR FMC63	APC	FMC63-derived human CD19 CAR	130-127-344	REA1297	Miltenyi Biotec	1:200