Molecular Therapy
Methods & Clinical Development
Brief report

CAR-NK cells derived from cord blood originate mainly from CD56$^-$CD7$^+$CD34$^-$HLA-DR$^-$Lin$^-$ NK progenitor cells

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Cord blood (CB)-derived chimeric antigen receptor (CAR)-natural killer (NK) cells targeting CD19 have been shown to be effective against B cell malignancies. While human CD56$^+$ NK cells can be expanded *in vitro*, NK cells can also be differentiated from hematopoietic progenitor cells. It is still unclear whether CAR-NK cells originate from mature NK cells or NK progenitor cells in CB. Here, we determined that CAR-NK cells were predominantly derived from CD56$^-$ NK progenitor cells. We first found that substantial numbers of CD19 CAR-NK cells were produced from CD56$^-$ CB mononuclear cells after *in vitro* culture for 2 weeks. Single-cell RNA sequencing analysis of CD56$^-$CD3$^-$CD14$^-$CD19$^-$ CB mononuclear cells revealed that these cells could be subdivided into three subpopulations based on the expression of CD34 and human leukocyte antigen (HLA)-DR. NK cells originated primarily from CD34$^-$HLA-DR$^-$ cells. In addition, among the CD34$^-$HLA-DR$^-$ cells, only CD7$^+$ cells could differentiate into NK cells. These results indicate that CD56$^-$CD7$^+$CD34$^-$HLA-DR$^-$ lineage marker (Lin)$^-$ cells are the major origin of human CB-derived CAR-NK cells, indicating the importance of developing methods to enhance the quality and quantity of NK cells produced from these NK progenitor cells.

INTRODUCTION

Autologous chimeric antigen receptor (CAR) T cell therapy has demonstrated remarkable efficacy in patients with B cell leukemia/lymphoma and multiple myeloma.[1–4] However, CAR T cell therapy is extremely expensive. In addition, several weeks are needed to produce CAR T cells from autologous T cells. Natural killer (NK) cells do not induce graft-versus-host disease when infused into allogenic donors,[5] making them attractive candidates as a source for universal cellular immunotherapy.[6] This approach allows for the creation of off-the-shelf products that can be employed for allogeneic recipients. CARs can be expressed on NK cells to reprogram their specificity toward a particular target.[7–10] NK cells derived from various sources, such as induced pluripotent stem cells,[11] cord blood (CB),[7,8] or NK cell lines,[12,13] are utilized in the generation of CAR-NK cells.

CB-derived CAR-NK cells targeting CD19 have been shown to be effective against B cell malignancies in clinical trials.[8,14] At present, CB-derived CAR-NK cells targeting various other molecules are being tested clinically.[5,6] Purified CD56$^+$ NK cells can be expanded by stimulation with K562-based feeder cells and interleukin (IL)-2.[7,15] It is also known that human NK cells can be differentiated from hematopoietic progenitor cells *in vitro*.[16–23] CD3-, CD14-, and CD19-depleted CB mononuclear cells (MNCs), which include many immature hematopoietic cells, were used to establish CAR-NK cells[8] in some clinical trials. It remains unclear whether CB-derived CAR-NK cells originated from mature NK cells or NK progenitor cells. In this study, we aimed to clarify this issue to improve methods for generating CAR-NK cells from CB cells.

RESULTS

CAR-NK cells originate predominantly from CD56$^-$ NK progenitor cells in human CB

CAR-NK cells that targeted CD19 and secreted IL-15 were established according to previous reports[7,10] (Figure 1A). We tested CB cells from

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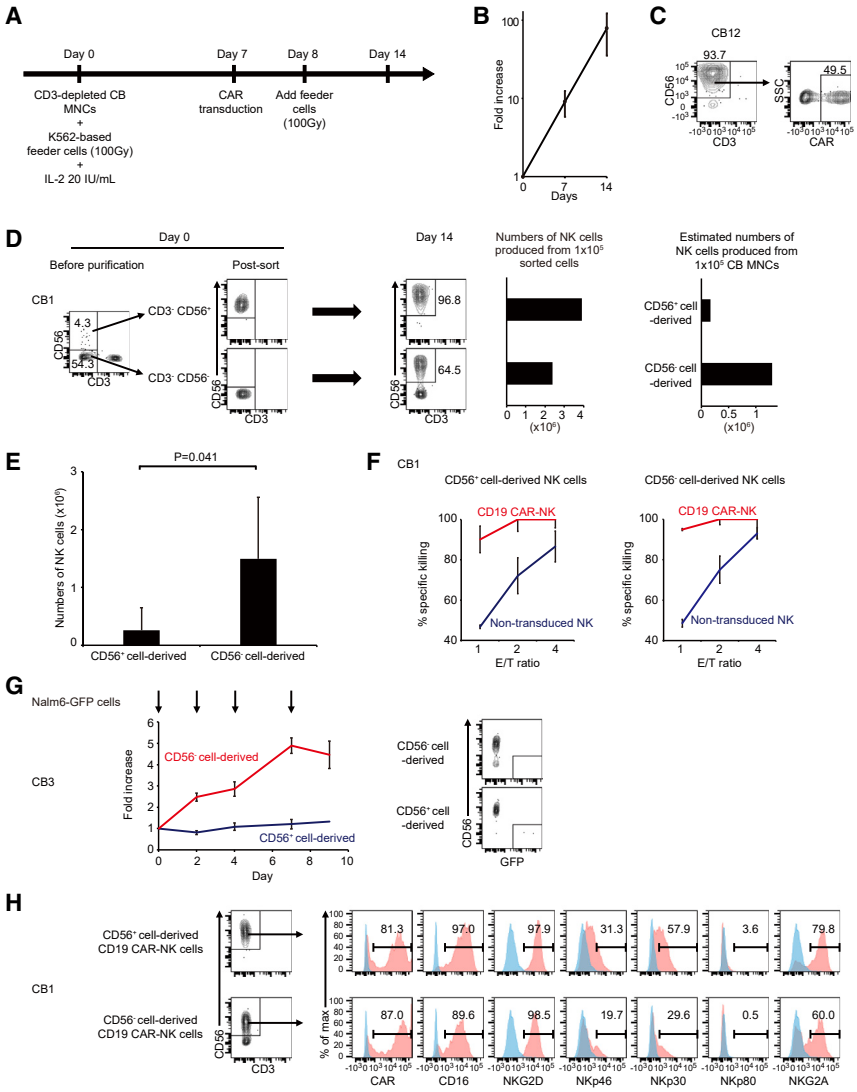

Figure 1. CAR-NK cells derived from CB originate predominantly from CD56⁻ NK progenitor cells

(A) A scheme showing the protocol to produce CD19 chimeric antigen receptor (CAR)-natural killer (NK) cells derived from cord blood (CB) mononuclear cells (MNCs). (B) Increase in $CD3^-CD56^+$ NK cell numbers during *in vitro* culture ($n = 6$). (C) Representative flow cytometric analysis of the expression of CD56, CD3, and CAR in CD19 CAR-NK cells. (D) (Left) Flow cytometry profiles and percentages of $CD3^-CD56^+$ NK cells produced from sorted $CD3^-CD56^+$ or $CD3^-CD56^-$ CB cells after 14 day culture shown in (A). (Middle) Numbers of NK cells produced from 1×10^5 $CD3^-CD56^+$ or $CD3^-CD56^-$ CB MNCs. (Right) Numbers of $CD56^+$ cell- or $CD56^-$ cell–derived NK cells produced from 1×10^5 CB MNCs were estimated by multiplexing the number of NK cells yielded from 1×10^5 $CD3^-CD56^+$ or $CD3^-CD56^-$ cells with their frequency in CB MNCs. A representative result of experiments using K562-4-1BBL-mbIL-15 cells as feeder cells is shown. Results from other CB samples are shown in Figure S1. Results of the experiments using other feeder cells are shown in Figures S2 and S3. (E) Estimated numbers of $CD56^+$ cell- or $CD56^-$ cell-derived NK cells yielded from 1×10^5 CB MNCs. Summary of the results from 5 CB samples is shown. (F) ^{51}Cr release assay for measuring specific lysis of Raji B cell lymphoma cells by CD19 CAR-NK cells or non-transduced NK cells produced from $CD3^-CD56^+$ or $CD3^-CD56^-$ cells. Representative results from experiments with four different donors are shown. (G) (Left) Proliferation of CD19 CAR-NK cells derived from $CD56^-$ or $CD56^+$ CB cells in response to repeated stimulation with GFP-expressing Nalm6 cells (effector:target [E/T] ratio = 3). (Right) Flow cytometric analysis 48 h after the fourth challenge with GFP-expressing Nalm6 cells. (H) Flow cytometric analysis of the expression of CAR or NK cell receptors in NK cells derived from $CD56^+$ or $CD56^-$ CB MNCs. Representative results from three independent experiments are shown. Results of other samples are shown in Figure S4D. The feeder cells used were as follows: K562-4-1BBL-mbIL-15 cells in (B)–(E), K562-4-1BBL-mbIL-15-mbIL-21 cells in (F) and (H), and K562-4-1BBL-mbIL-21 cells in (G). Throughout the figure, error bars represent the standard deviation (SD).

30 different donors and found that NK cells were efficiently expanded from 14 samples, and these samples were used in this study. After 2 weeks of culture, the number of $CD3^-CD56^+$ NK cells increased by 78.7-fold (Figure 1B). CD19 CAR was efficiently transduced into NK cells (Figure 1C).

$CD3^-CD56^+$ NK cells or $CD3^-CD56^-$ cells were purified by fluorescence-activated cell sorting (FACS) and separately subjected to 2 week culture using K562 cells transduced with 4-1BBL and membrane-bound (mb)IL-15 as feeder cells to generate NK cells (Figure 1D). NK cells were produced from not only $CD3^-CD56^+$ NK cells but also $CD3^-CD56^-$ NK cells (Figure 1D). For example, in a CB sample, 1×10^5 $CD3^-CD56^+$ cells or $CD3^-CD56^-$ cells yielded 3.9×10^6 or 2.4×10^6 $CD56^+CD3^-$ NK cells, respectively, after 2 weeks of culture. Since the frequencies of $CD56^+CD3^-$ cells and $CD56^-CD3^-$ cells in this CB sample were 4.3% and 54.3%, respectively, these results suggest that 1.7×10^5 (=$3.9 \times 106 \times 0.043$) $CD56^+$ cell-derived NK cells and 1.3×10^6 (=$2.4 \times 106 \times 0.543$) $CD56^-$ progenitor cell-derived NK cells could be produced from 1.0×10^5 CB MNCs (Figure 1D). The results of the same analysis using other CB samples ($n = 5$; Figure S1) also showed that a significantly higher number of CAR-NK cells were produced from $CD3^-CD56^-$ NK progenitor cells than from $CD3^-CD56^+$ NK cells (Figure 1E). In addition, NK cells were predominantly produced from $CD3^-CD56^-$ NK progenitors in 4 of 6 experiments using K562 feeder cells expressing 4-1BBL, mbIL-15, and mbIL-21 and also in 2 of 2 experiments using those expressing 4-1BBL and mbIL-21 (Figures S2 and S3). Both $CD56^+$ cell-derived NK cells and $CD56^-$ cell-derived NK cells showed

significant cytotoxicity upon co-culture with $CD19^+$ B cell lymphoma cells (Figures 1F, S4A, and S4B). $CD56^-$ cell-derived NK cells showed greater proliferative potential in response to repeated antigen stimulation compared with $CD56^+$ cell-derived NK cells (Figures 1G and S4C). In addition, both $CD56^-$ cell-derived NK cells and $CD56^+$ cell-derived NK cells could eradicate GFP-expressing Nalm6 cells even after the fourth round of tumor challenge (Figures 1G and S4C). The expression levels of receptor molecules such as CD16, NKG2D, NKp46, NKp30, NKp80, and NKG2A in the $CD56^-$ cell-derived CAR-NK cells were comparable with those in the $CD56^+$ cell-derived CAR-NK cells (Figures 1H and S4D).

CB-derived NK cells originate predominantly from $CD7^+CD34^-HLA\text{-}DR^-CD56^-Lin^-$ cells

Single-cell RNA sequencing (RNA-seq) analysis of $CD56^-CD3^-CD14^-CD19^-$ CB MNCs, which had the potential to produce $CD56^+CD3^-$ NK cells (Figure 2A), revealed that these cells were subdivided into five subpopulations (Figures 2B, 2C, S5A, and S5B). The first was enriched with cells expressing $CD34^+$ hematopoietic stem and progenitor cells. The second and third were enriched with cells showing high expression of HLA-DR but not FCGR3A, presumably dendritic cells. The fourth was enriched with $HLA\text{-}DR^+FCGR3A^+$ monocytic cells. The fifth was enriched with cells that did not express either CD34 or HLA-DR; this population included cells expressing killer cell lectin-like receptor B1 (KLRB1), IL2RB, killer cell lectin-like receptor K1 (KLRK1), or ETS1,[24] suggesting that it was enriched with cells committed to the NK cell lineage.[23]

Consistent with the results of single-cell RNA-seq analysis, the $CD56^-CD3^-CD14^-CD19^-$ (hereafter called $CD56^-$ lineage marker $[Lin]^-$) CB MNCs were subdivided by flow cytometric analysis into the following three distinct cell populations: $CD34^-HLA\text{-}DR^+$, $CD34^+HLA\text{-}DR^{+/-}$, and $CD34^-HLA\text{-}DR^-$ (Figures 2D and S5C). These three populations were purified and separately cultured to generate NK cells. After 14 days of culture, NK cells were produced almost exclusively from $CD34^-HLA\text{-}DR^-$ cells (Figures 2D, 2E, and S5C).

Single-cell RNA-seq analysis showed that CD7-expressing cells were almost exclusively detected in the $CD34^-HLA\text{-}DR^-$ cell population, which was subdivided into $CD7^+$ and $CD7^-$ populations (Figure S6A). In addition, among several cell surface antigens that were reported to be expressed on NK cells or NK progenitor cells,[23] CD7 expression could be used to subdivide $CD34^-HLA\text{-}DR^-CD56^-Lin^-$ cells into 2 distinct subpopulations (Figure S6B), which were then separately cultured to generate NK cells. NK cells were produced from $CD7^+$ cells but not from $CD7^-$ cells (Figures 2F, 2G, and S6C). CD19 CAR-NK cells generated from $CD7^+CD34^-HLA\text{-}DR^-CD56^-Lin^-$ cells showed significant cytotoxic potential against B cell leukemia/lymphoma cells (Figure 2H). $CD7^+CD34^-HLA\text{-}DR^-CD56^-Lin^-$ cells were also observed in peripheral blood mononuclear cells (PBMCs) (Figure S6D). Taken together, these results indicate that $CD7^+CD34^-HLA\text{-}DR^-CD56^-Lin^-$ cells are the major origin of CB-derived NK cells.

DISCUSSION

In this study, we demonstrated that CAR-NK cells derived from CB originated predominantly from $CD56^-$ NK progenitor cells rather than from $CD56^+$ mature NK cells. We also showed that $CD56^-$ cell-derived CAR-NK cells demonstrated greater potential to proliferate in response to repeated antigen stimulation than $CD56^+$ cell-derived CAR-NK cells. Moreover, we identified $CD7^+CD34^-HLA\text{-}DR^-CD56^-Lin^-$ CB cells as NK progenitors. These cells are likely to differentiate from the previously reported $CD7^+CD34^+$ NK progenitors.[25,26] We intend to conduct a thorough analysis of the characteristics of $CD7^+CD34^-HLA\text{-}DR^-CD56^-Lin^-$ NK progenitors and develop methods to expand and efficiently differentiate these cells into NK cells to improve the quality and quantity of CB-derived CAR-NK cells.

Previous studies have reported the differentiation of NK cells from $CD34^+$ cells in human bone marrow or CB following culture with various cytokines, including IL-15.[23,25–27] Some studies have reported that co-culture with stromal cells is needed to produce NK cells from $CD34^+$ cells.[16,17,19,28] However, these studies indicated that 3–5 weeks of culture were required to yield NK cells, with low efficiencies in most cases. We discovered that the $CD34^-$ fraction of CB cells contained NK cell-committed progenitor cells that led to the production of a substantial number of NK cells after just 2 weeks of culture.

We need to optimize our culture method to efficiently produce CAR-NK cells from CB. The quality of CB cells is highly variable[14] and must be carefully assessed when selecting the source of CAR-NK cells. Crosstalk between NK progenitor cells and myeloid or B cells that are part of the $CD56^-$ cell population may enhance or suppress NK cell production from $CD56^-$ cells. In some samples in our experiments, $CD56^-CD3^-$ cells persisted after 14 day culture of $CD56^-$ CB MNCs, suggesting that more NK cells can be obtained by extending the culture time. In addition, small numbers of T cells were generated from $CD3^-CD14^-CD19^-CD34^+HLA\text{-}DR^{+/-}$ CB MNCs in 1 of 3 samples, suggesting that T cell generation must be carefully monitored during CAR-NK cell production.

MATERIALS AND METHODS

Cells

CB cells were obtained from the Kinki Cord Blood Bank and the Hyogo Cord Blood Bank after informed consent was obtained from donors. This study was approved by the institutional review boards of the Osaka University Graduate School of Medicine, Kinki Cord Blood Bank, and Hyogo Cord Blood Bank. Raji cells were purchased from the Japan Collection of Research Bioresources Cell Bank. K562 cells expressing mbIL-15 and 4-1BBL (K562-4-1BBL-mbIL-15 cells)[10] were kindly provided by St. Jude Children's Research Hospital. We established K562-4-1BBL-mbIL-21 cells and K562-4-1BBL-mbIL-15-mbIL-21 cells by retrovirally transducing K562 cells (ATCC) with 4-1BBL, mbIL-15, or mbIL-21 complementary DNA (cDNA). Expression of 4-1BBL, mbIL-15, and mbIL-21 was examined by flow cytometry using anti-4-1BBL (BioLegend, 5F4), anti-IL-15 (R&D, #34559), and anti-IL-21 (BioLegend, 3A3-N2)

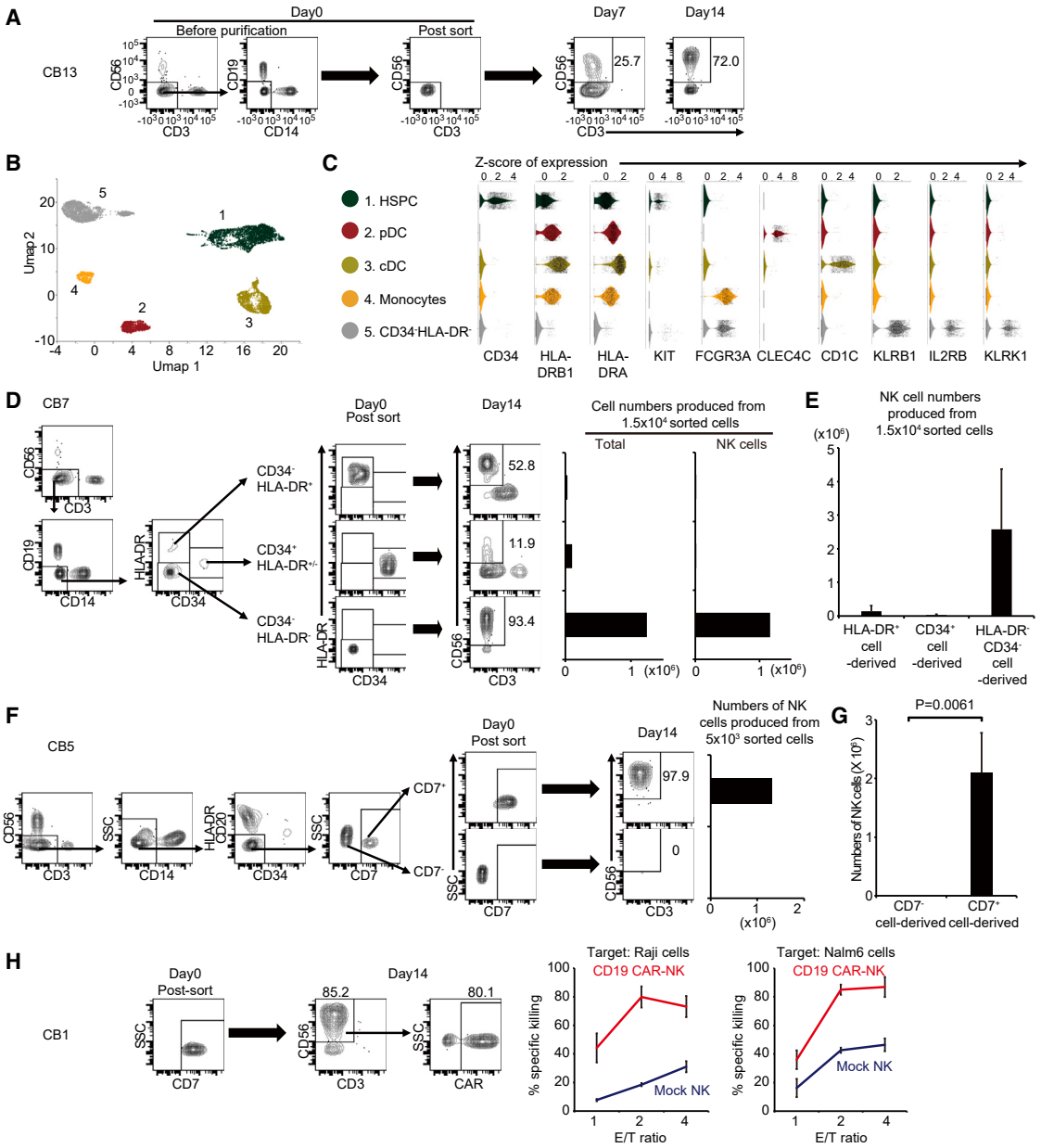

Figure 2. NK cells derived from CB originate predominantly from CD7⁺CD34⁻HLA-DR⁻CD56⁻Lin⁻ cells

(A) Flow cytometry profiles and percentages of CD3⁻CD56⁺ NK cells produced from sorted CD3⁻CD56⁻CD14⁻CD19⁻ CB MNCs after 7 or 14 day culture shown in Figure 1A. (B) Uniform manifold approximation and projection (UMAP) embedding of single-cell RNA-seq data for CD3⁻CD56⁻CD14⁻CD19⁻ CB MNCs (n = 7,254 cells from three CB samples). (C) Violin plots showing the expression of cell surface markers in each cell population. HSPC, hematopoietic stem or progenitor cells; pDC and cDC, plasmacytoid and conventional dendritic cells, respectively. (D and E) Flow cytometry profiles and percentages of CD3⁻CD56⁺ NK cells produced from CD34⁻HLA-DR⁺, CD34⁺HLA-DR$^{+/-}$, or CD34⁻HLA-DR⁻ cells purified from CD3⁻CD56⁻CD14⁻CD19⁻ CB MNCs after 14 day culture. Numbers of total cells and NK cells produced from 1.5×10^4 sorted cells are plotted in bar graphs. Representative results from a CB sample are shown. Results from other samples are shown in Figure S5C. A summary of the results from three independent CB samples is shown in (E). (F and G) Flow cytometry profiles and percentages of CD3⁻CD56⁺ NK cells produced from CD7⁺ or CD7⁻ cells purified from CD34⁻HLA-DR⁻CD56⁻Lin⁻ CB MNCs. Numbers of NK cells produced from the indicated populations are plotted in bar graphs. Representative results from a CB sample are shown in (F). Those from other samples are shown in Figure S6C. A summary of the results from three independent CB samples is shown in (G). (H) (Left) Flow cytometry profiles of CD19 CAR-NK cells derived from CD7⁺CD34⁻HLA-DR⁻CD56⁻Lin⁻ CB MNCs. (Right) ^{51}Cr release assay for measuring specific lysis of Raji or Nalm6 cells by CD19 CAR-NK cells or non-transduced NK cells produced from CD7⁺CD34⁻HLA-DR⁻CD56⁻Lin⁻ CB MNCs. In all experiments except for (H), K562-4-1BBL-mbIL-15 cells were used as feeder cells. In (H), K562-4-1BBL-mbIL-15-mbIL-21 cells were used. Throughout the figure, error bars represent the standard deviation (SD).

monoclonal antibodies (mAbs). Single-cell cloning was performed to establish the feeder cells used in the experiments.

Retrovirus production

pEQ-PAM3(-E) and pRDF plasmids were gifts from Toshio Kitamura (The University of Tokyo) and Keiichiro Mihara (Fujita Health University), respectively. cDNAs of the variable regions of the κ light chain and the heavy chain of the anti-CD19 mAb FMC63[29] fused with CD28, CD3ζ, and T2A-IL-15 cDNAs were inserted into the retroviral vector. To generate viral supernatants, 293T cells were co-transfected with the retroviral vector, pEQ-PAM3(-E), and pRDF using Lipofectamine 2000 reagent (Thermo Fisher Scientific).

Generation of CAR-NK cells

T cells were depleted from CB MNCs using CD3 MicroBeads (Miltenyi Biotec). T cell-depleted CB MNCs (3.75×10^5/mL) were co-cultured with 100 Gy-irradiated K562-4-1BBL-mbIL-15 cells (2.5×10^5/mL) in RPMI 1640 medium supplemented with 10% fetal bovine serum and 20 IU/mL IL-2. After 1 week, retroviruses carrying CD19 CAR-T2A-IL-15 cDNA were transduced into CB-derived NK cells using RetroNectin (Takara Bio). The cells were then re-stimulated with 100 Gy-irradiated K562-4-1BBL-mbIL-15 cells, cultured for an additional week.

Flow cytometry and cell sorting

mAbs used in this study are listed in Table S1. Flow cytometry and cell sorting were performed using a BD FACS Canto II or FACS Aria II (BD Biosciences) and analyzed with FlowJo software (BD Biosciences).

Cytotoxicity assays

Target cells (6×10^5) were labeled for 1.5 h with 25 μCi of [^{51}Cr] sodium chromate (PerkinElmer). Labeled target cells (1×10^4) were incubated with effector cells for 4 h. ^{51}Cr release in harvested supernatants was measured with a gamma counter. The percentage of specific lysis was calculated as previously reported.[30]

Cytotoxicity of CAR-NK cells was also assessed with a flow-cytometry-based cytotoxicity assay that used GFP-expressing Nalm6 cells as targets. Target cells (1×10^4) were incubated with effector cells at the indicated effector:target ratio. For tumor rechallenge, cells were washed and resuspended in the fresh medium containing the target cells.

Single-cell RNA-seq analysis

CB MNCs from three independent donors were stained with CD14, CD19, CD3, CD56, and one of three different TotalSeq-C anti-human hashtags (LNH-94; 2M2, Barcoded, BioLegend). $CD56^-CD3^-CD14^-CD19^-$ MNCs were sorted by FACS and then analyzed. A Chromium Next GEM Single Cell 5′ Library and Gel Bead Kit v.2 (10× Genomics) was used to construct a single-cell RNA-seq library. Libraries were sequenced on a NovaSeq 6000 platform in a 28 + 90-base paired-end mode to yield a minimum of 20,000 reads per cell for gene expression. The single-cell RNA-seq dataset was processed, explored, and visualized using a Cellenics community instance (https://scp.bioimage.net/) hosted by Biomage (https://biomage.net/). The data discussed in this publication have been deposited in the Gene Expression Omnibus[31] of the National Center for Biotechnology Information and are accessible through GEO Series accession number GEO: GSE253575 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE253575).

Statistical analysis

The unpaired two-tailed Student's t test was used to determine statistically significant differences between samples.

DATA AND CODE AVAILABILITY

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

T.W., Y.K., and N.H. designed the research study, performed experiments, analyzed data, and wrote the paper. S.I., M.Y., M.T., M.S., S. Kida, K.S., K.T., H.M., Y.U., H.K., K.F., J.F., T.U., S. Kusakabe, A.H., M.I., C.I., D.O., and A.K. performed experiments.

DECLARATION OF INTERESTS

N.H. has applied for a Japanese patent entitled "NK progenitor cells and method of producing NK cells using the progenitor cells" through the Osaka University Office for University-Industry Collaboration.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101374.

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