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Supplementary Information for

Memory-like NK cells armed with a neoepitope-specific CAR exhibit potent activity against NPM1 mutated acute myeloid leukemia.

Han Dong, James Dongjoo Ham, Guangan Hu, Guozhu Xie, Juliana Vergara, Yong Liang, Alaa Ali, Mubin Tarannum, Hannah Donner, Joanna Baginska, Robert Soiffer, Jerome Ritz, Laurie Glimcher*, Jianzhu Chen* and Rizwan Romee*

*Corresponding authors.
Email: rizwan_romee@dfci.harvard.edu, jchen@mit.edu, laurie_glimcher@dfci.harvard.edu.

This PDF file includes:

SUPPLEMENTAL MATERIALS AND METHODS

Cell line culture

OCI-AML3, K562, Jurkat and 293T cells were purchased from ATCC. OCI-AML2 cells were purchased from DSMZ. 293 T cells were adapted to suspension culture utilizing FreeStyle following the methods detailed in Bauler et al.[1] Cell lines were routinely tested for mycoplasma contamination. 293T cells were cultured in DMEM medium (Gibco) supplemented with 10% FBS (Life Tech and VWR), 2 mM L-Glutamine (Thermo Fisher Scientific) and 1% v/v Penicillin-Streptomycin solution (Life Technologies), namely DMEM10; all others were cultured in RPMI 1640 medium (Gibco) using the same supplements, namely RPMI10. Cell lines were cultured in incubator at 37°C with 5% CO2.

Construction of CAR lentiviral vectors

The sequence of CAR, consisting of the completely human anti-NPM1c scFv (clone: YG1)[2], the CD8a signal peptide sequence, extracellular hinge domain and transmembrane domain, the 4-1BB co-stimulatory domain, and the CD3ζ activation domain, was custom synthesized by Integrated DNA Technologies (IDT). The second fragment was synthesized by the same way consisting of self-cleavage P2A followed by either EGFP (P2A-GFP) or membrane-bound IL-15 (P2A-mb15). The pHIV vector (plasmid #21373) was doubly digested with the enzymes XbaI and ClaI. After gel purification of the vector backbone, the pHIV backbone, CAR fragment and the corresponding P2A-linked fragment were assembled basing on their overlap region at 5' and 3' termini using HiFi DNA Assembly Master Mix (New England BioLabs) according to the manufacturer's protocol. The resulting plasmids were sequenced using following sequencing primers: 5'-

GTTAGGCCAGCTTGGCACTTGATGT-3' (forward) and 5' AGGCACAATCAGCATTGGTAGCTG-3' (reverse). The plasmid with the correct sequence was named pHIV-CAR-GFP and pHIV-CAR-mb15, respectively.

Expansion of plasmids

A second-generation LV system was used in this study. All lentiviral plasmids including pCMV-ΔR8.91[3], pAdVAntage (Promega Corporation), pCMV-BaEV or pCMV-VSV-G, along with the CAR lentiviral vectors pHIV-CAR-GFP or pHIV-CAR-mb15 (and in Fig. S5 pHIV-CAR-mb15/Ra), were transformed into Stbl3 chemically competent *E. coli* (Thermo Fisher C737303) and propagated by expansion in LB broth supplemented with 100mg/mL ampicillin. Plasmids were purified via Endo-free Maxi Prep (Qiagen #12362).

BaEV-pseudotyped lentivirus packaging

Suspension-adapted 293T cells were cultured in sterile Erlenmeyer flasks under constant orbital shaking at 140 rpm. Lentivirus was generated by transfecting suspension-adapted 293T cells with pHIV-CAR-GFP (or pHIV-CAR-mb15), pCMV-BaEV, pCMV-ΔR8.91, and pAdVAntage plasmids. Lentiviral supernatant was collected at 48 and 72 hours post-transfection and filtered through a 0.45 μm filter. Filtered supernatant was centrifuged overnight at 8000xg, 4℃. After removing the supernatant, the viral pellet was resuspended with PBS and frozen at 80℃ for storage.

Titering of BaEV-pseudotyped lentivirus

Functional lentiviral titers were calculated using a FACS-based measurement of CAR expression in transduced Jurkat cells. Briefly, 500,000 Jurkat cells were seeded at 1 million/mL in 24-well plates with polybrene at 10 μg/mL. Different volumes of lentivirus were added to each well in duplicate, then spinfected at 1000xg for 1.5 hour at 37°C with brake low. At 72 hours post-transduction, the % CAR-positive cells was measured by flow cytometric analyses of GFP and/or scFv surface expression using Alexa Fluor 647 AffiniPure F(ab')$_2$ Fragment Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch) with background subtracted from untransduced controls. Finally, assuming MOI=1 for Jurkat cells, the functional lentiviral titer was back calculated using the following formula: [5E5* %CAR-positive/virus volume (μL)] TU/μL.

PBMC Isolation and preparation of CIML NK cells

Whole blood was collected from anonymous healthy donors under the blood collection protocol approved by the Institutional Review Board of Brigham and Women's Hospital. Healthy donor PBMC were isolated by Ficoll centrifugation and NK cells were subsequently purified using the NK Cell Isolation Kit (Miltenyi Biotec), or directly using RosetteSep immunodensity cell separation (Stem Cell Technologies). After purity check by flow cytometry (>95% $CD56^+$ $CD3^-$), the isolated human primary NK cells were incubated overnight (12-16 hours) at 37°C in RPMI10 medium supplemented with rhIL-15 (1ng/mL, StemCell Technologies) to generate conventional NK (cNK) cells. CIML NK cells were generated by supplementing the RPMI10 medium with a combination of rhIL12 (10 ng/mL, StemCell Technologies) + rhIL-15 (50 ng/mL, StemCell Technologies) + rhIL-18 (50 ng/mL, Life Technologies) as described before.[4, 5] Fresh PBMC were used for all

experiments in this study, though CIML NK cells generated from cryopreserved PBMC also showed comparable functional activity.

Transduction of human primary CIML NK cells using BaEV-pseudotyped lentivirus

One day prior to transduction, we coated nontreated, cell-culture grade 24-well plates with 20 μg/ml RetroNectin (TaKaRa Bio) in PBS at 500 μL/well, incubated overnight at 4 ℃. The coated plate was then blocked with 2% BSA in PBS at room temperature for 30 minutes and washed twice with PBS before using. For BaEV lentiviral transduction, cNK or CIML NK cells generated by cytokine stimulation the night before as described above were first washed twice with RPMI10 medium to remove residual cytokines and with a slow spinning at 700 rpm, 10 minutes to remove possible cell debris, and then resuspended at 1×10^6 cells/200 μL/well in RPMI10 medium with 500 U/mL rIL-2 and 1ng/ml rIL-15. Mixture (300 μl) of Vectofusin-1 and lentivirus was added into 200 μL cell suspension for transduction in one well of 24-well plate. Final concentration of Vectofusin-1 was 10 μg/mL and final MOI=10 for the BaEV-pseudotyped lentivirus. After a spinfection at 1000xg, 32℃ for 1.5 hours, the cells were cultured with the lentivirus for 48 hours at 37℃ with 5% CO2. The cell culture medium was then exchanged with fresh NK MACS medium (Miltenyi Biotec) supplemented with 5% Human AB serum, 500 U/mL rIL-2 and 1ng/ml rIL-15. We added the fresh medium every other day to support the culture expansion and transferred cells from 24-well plates to 12- or 6-well plates around day 7 post transduction. NK cell concentration was kept between 0.5 to 2 million/ml. After 3 days, CAR-expression on NK cells were determined using Alexa Fluor 647 AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch). CIML NK cells were expanded for

4-6 days before *in vitro* experimentation or 7-10 days before *in vivo* experimentation. CIML NK cells were expanded for 2-4 weeks to assess their long-term survival and function (Fig. 1i and S2).

Quantitative real-time PCR

NK cells were either enriched with magnetic beads (Miltenyi Biotec) or sorted using FACSAria II cell sorter (BD Biosciences) to >95% purity, and RNA was isolated using the RNeasy RNA Purification Mini (for $5*10^5$ cells or beyond) or Micro (for <5*105 cells) kit (Qiagen) according to the manufacturer's protocol. From purified RNA, complementary DNA was synthesized using the reverse-transcriptase kit qScript cDNA synthesis kit (Quanta Biosciences). q-rtPCR was performed in triplicates in a 384-well plate using Taqman Probes (Themo Fisher)-based detection on an ABI QuantStudio 6 Flex qrtPCR machine. For the analysis of mRNA abundance, the derived values were normalized to those of β-Actin (β-ACTIN for human materials) using a detaCt method. Taqman probe information for SLC1A5 (ASCT2), SLC1A4 (ASCT1), IL15 and bACT are in Table S1.

Intracellular cytokine staining

CAR transduced or untransduced human primary CIML NK cells were cultured for 4-6 days after spinfection. Cells were then harvested and stimulated with either AML target cells OCI-AML3 ($NPM1c^+$ $HLA\text{-}A2^+$), OCI-AML2 (NPM1-wildtype $HLA\text{-}A2^+$) or NK-sensitive K562 cells (HLA^-) in a 1:1 ratio, at 4×10^5 cells/150 µL/well in 96-well round bottom plates. RPMI10 medium was used in the presence of degranulation marker antibody anti-CD107a (Biolegend) and cells were incubated at 37 °C, 5% CO_2 for 1 hour before the

protein transport inhibitor monensin (BD Biosciences) and brefeldin A (BioLegend) were applied. After another 4-5 hours of incubation, cells were then washed, stained with Live/Dead Fixable Dead Cell Stain (Thermo Fisher) and anti-Human IgG(H+L), followed by surface staining for anti-CD56 and CD3, then fixed, permeabilized (BD), and intracellularly stained with anti-IFNγ (BioLegend). Cells were analyzed by a CytoFLEX (Beckman Coulter) flow cytometer, and live $CD56^{+}CD3^{-}CAR^{+}$ lymphocytes were gated for further analysis. All flow cytometric samples were analyzed using FlowJo (Tree Star) software.

Flow cytometric quantifications of ASCT2 and LDL-R

Conventional or CIML NK cells were stained with fixable viability dye and surface markers as described above (in some experiments, NK cell maturation markers were also included such as NKG2A, CD57, CD56 and KIRs), and then fixed according to the manufacture's protocol before stained with anti-ASCT2 rabbit monoclonal antibody (clone D7C12, Cell Signaling Technology) or the matched isotype control antibody (# 3900, Cell Signaling Technology). Then a secondary anti-rabbit antibody was used to allow the flow cytometric analyses. LDL receptor (LDL-R) surface expression was quantified by using Alexa Fluor 647-conjugated anti-human LDL-R Antibody (# FAB2148R, R&D Systems) and its isotype control antibody (#IC002R, R&D Systems).

Phosflow

Flow cytometric quantifications of pSTAT5 and pS6 were performed per the manufacture's protocol (BD Biosicences) by using Phosflow Fix buffer I, Perm Buffer III before staining

with conjugated antibodies for anti-pSTAT5 [Clone 47/stat5p(Y694)] and S6 (pS232/pS236, Clone N7-548) in Pharmingen Stain Buffer.

Immunoblots

Purified NK cells (purity >97%) were collected directly into 20% trichloroacetic acid buffer. After sorting, the concentration of trichloroacetic acid was adjusted to 10% before incubation on ice for 30 min to precipitate proteins. The homogenates were centrifuged at 13,000 rpm for 10 min at ℃, and the supernatant was carefully removed. The pellets were washed twice with acetone and then solubilized in solubilization buffer (containing 9 M urea, 2% Triton X-100 and 1% dithiothreitol) mixed with LDS buffer (Invitrogen). The samples were heated at 70℃ for 10 min and protein concentrations determined using a BCA protein assay kit (Thermo Fisher Scientific). Equivalent amount of protein was separated by SDS-PAGE (Bis–Tris gel, Invitrogen) and transferred onto polyvinylidenedifluoride membranes following the standard protocol. Anti-IL15 (CF807785, OriGene), anti-β-Actin (# 4967S, Cell Signaling Technology) and goat anti-rabbit secondary antibodies conjugated with HRP (Thermo Fisher Scientific) were used. SuperSignal West Pico and Femto chemiluminescent substrates (Thermo Fisher Scientific) were used to image blots in a ChemiDoc MP Imaging System instrument (BioRad).

Cytotoxicity of CAR-NK cells *in vitro*

To assess the ability of CAR-NK cells to kill target cells, CAR-NK cells and untransduced control cells from the same donors were incubated with luciferase-expressing OCI-AML3 or OCI-AML2 cells at indicated effector: target ratios. 20 hours after co-culture, luciferase

assays (Promega) were performed according to the manufacturer's protocol. The reduction in target cell numbers due to NK cell-mediated killing was quantified by the endpoint luciferase signals normalized to the corresponding control wells where the culture only contained target cells. E:T ratios were calculated using total NK cells, not GFP^+ or CAR^+ NK cells.

To quantify the early apoptosis of target cells by flow cytometry, we pre-labeled target cells with CellTrace dye (Thermo Fisher) before setting up similar co-cultures with NK cells. Cells were harvested after 4 hours, and routine viability and surface marker staining was performed as described above. Cell samples were then incubated with anti-Annexin V in Annexin V Binding Buffer (in eBioscience Annexin V Apoptosis Detection Kit) according to the manufacturer's protocol before analysis by a CytoFLEX flow cytometer.

Cytotoxicity of CAR CIML NK cells on $NPM1c^+$ $HLA\text{-}A2^+$ primary AML blasts *in vitro*

Anti-NPM1c CAR and untransduced control CIML NK cells were pre-labeled with CFSE while $NPM1c^+HLA\text{-}A2^+$ primary AML blasts from three donors were pre-labeled with CellTrace Violet (CTV) according to the manufacturer's protocol (Invitrogen). NPM1c CAR CIML NK cells or untransduced control cells were incubated with AML blasts at the indicated ratios for 20 hrs. The absolute numbers of viable AML blasts were quantified by flow cytometry as $DAPI^-$ CTV^+ $CFSE^-$ cells. The percentages of lysis of tumor cells at different effector-to-target ratios were calculated using the absolute numbers of viable AML blasts from the control cultures with AML cells only as the denominator.

***In vivo* testing of CIML CAR-NK cells in AML xenograft models in NSG mice**

8- to 12-week-old NOD-*scid* IL2Rγc^{-} (NSG) mice were purchased from the Jackson Laboratories and housed in the specific pathogen-free (SPF) vivarium at the Longwood Center (Dana-Farber Cancer Institute). Both female and male mice were evaluated in this study and each experimental cohort consisted of age- and gender-matched mice. All animal work described in this study complied with local animal ethical and welfare standards. All experiments with mice were conducted approved by the Institutional Animal Care and Use Committee at Dana-Farber Cancer Institute. Briefly, NSG mice were irradiated with sublethal dose (250 cGy) and 2 days later, luciferase-expressing OCI-AML3 cells (5×10^5) in 200 μL PBS were injected intravenously (IV) into NSG mice. After 4 days, 1×10^6 untransduced or transduced CIML NK cells (containing about 500,000 CAR-expressing cells) were injected IV into the tumor-bearing mice. Mice were randomly allocated to different treatment groups. Bioluminescence imaging (BLI) was performed in a blinded manner at the indicated time points using a Xenogen IVIS-200 Spectrum camera.

To analyze tumor cells and NK cells by flow cytometry, mice were euthanized two to three weeks post NK cell adoptive transfer, and blood, spleen, bone marrow and liver were harvested. About 200 μL of blood was collected into microcentrifuge tube containing 1ml of PBS with 4 mM EDTA. Cells were pelleted by centrifugation at 1,500 rpm for 5 min and resuspended with 1 mL of ACK Lysis Buffer (Lonza Bioscience) by gently pipetting the mixture up and down and kept at room temperature for 5 min. The cells were centrifuged at 1,500 rpm for 5 min and then resuspended in FACS buffer. Prior to organ

harvesting, liver was perfused with 5 mL PBS through superior mesenteric vein. Spleen and liver were mechanically disrupted by pressing them through a 70 mm strainer using a syringe plunger. The lymphocytes from disrupted liver were isolated by using 40%: 60% Percoll (Sigma Aldrich) gradient centrifugation. Bone marrow cells were collected from the bilateral femurs by flushing with 5 ml of cold PBS. Single-cell suspensions were prepared and lysed of red blood cells. Cells were washed with FACS buffer, centrifuged at 1,500 rpm, 4℃ for 5 min, resuspended with FACS buffer and counted with trypan blue staining by a Countess II automated cell counter (Life Technologies).

For flow cytometry analysis, about 1 million cells were aliquoted and mixed with 20 μL human serum to block Fc receptor for 5 min. The cells were stained with Live/Dead Fixable Dead Cell Stain (Thermo Fisher) and anti-Human IgG(H+L) on ice for 1 hour, followed by the routine surface marker staining with the following conjugated antibodies at 4℃ for 30 min in the dark: anti-mCD45 (BD Biosciences), anti-hCD45 (BioLegend), anti-hCD56 (BioLegend), anti-hCD33 (BioLegend), Cells were washed with FACS buffer and resuspended in FACS buffer. Flow cytometry analysis was performed using a CytoFlex cytometer (Beckman Coulter).

Single-cell RNA-sequencing analyses

CIML NK cells were transduced with CAR-mb15 at MOI of 5 and then cultured for 7 days. One day before the analyses, CIML NK cells (mixture of CAR^+ and CAR^-) were flow-sorted to remove dead cells/debris. CIML NK cells were then cultured alone or co-cultured with OCI-AML3 target cells in 1:2 ratio for 24 hours before harvesting for library

preparation and sequencing. Cells were loaded onto 10X chromium machine (10X Genomics, San Francisco, CA) and run through library preparation using the Chromium Next GEM Single Cell 3' kits (10X Genomics). The single-cell cDNA libraries were sequenced by NovaSeq S4 flowcell (Illumina). Raw sequences were demultiplexed, aligned, filtered, barcode counting, unique molecular identifier (UMI) counting with Cell Ranger software v3.1 (10X Genomics) to digitalize the expression of each gene for each cell. The analysis was performed using the Seurat 3.0 package.[6] We first processed each individual data set separately prior to combining data from multiple samples. The outlier cells with extreme low number (< 500) or high number (> 5,000) of gene features as doublets, or low total UMI (< 1,000) and high mitochondrial ratio (> 15%) from each data set were removed. Subsequently, samples were combined based on the identified anchors for the following integrated analysis. To mitigate the effects of cell cycle heterogeneity, cell cycle phase scores based on canonical markers were calculated and then the gene expression were adjusted by subtracting ('regress out') this source of heterogeneity from the original data.[7] We ran principal component analysis (PCA) and used the first 15 principal components (PCs) to perform tSNE clustering.

To compare the difference of CAR^+ and CAR^- CIML NK cells, CAR^+ cells were defined with expression of the full CAR sequence >=0.1 while CAR^- cells with expression <=0. To demonstrate the difference of subpopulations, CAR^+ and CAR^- cells were clustered separately, clusters were matched based on the correlation similarities of global gene expression. To investigate CAR^+ NK response to tumor cells, CAR^+ cells were isolated from the co-cultured samples based on the expression of full CAR sequence >=0. Based

on previous publications, maturation signature includes NCAM1 (CD56), FCGR3A (CD16), B3GAT1 (CD57), KLRC1, KLRC2; cytotoxicity signature includes: LAMP1, GZMB, GZMA, PRF1, CD69, IL2RA, NCR1, NCR2, NCR3, KLRK1, ICOS, CD226; inhibitory signature includes: TIGIT, HAVCR2, KLRB1, CXCR4, KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2; KIR signature (inhibitory) includes: KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2. Differential expression analyses were performed to identify the genes significantly upregulated in each cluster compared with all other cells by setting $\log_2$ fold-change>=0.2 and P-value<0.05. For gene sets representing specific cellular functions or pathways, we performed functional enrichment analysis with the biological process of Gene Ontology by the online tool DAVID.[8]

Mass cytometry and data analyses

CAR CIML NK cells generated from three healthy PBMC donors were assessed by mass cytometry using a custom NK cell function panel consisting of 38 markers (see Table S2). In-house conjugations of antibodies were performed with Maxpar labeling kit per manufacturer instructions (Fluidigm). All antibodies were used per the manufacturer's recommendation.

For mass cytometry staining, cell samples were harvested into a conical tube with warm 15% RPMI medium, counted using AO/PI and pelleted by centrifugation at 400xg for 5 minutes. Cells were then incubated in 103Rh viability stain for 15 minutes before resuspending in CyFACS and plated at an average of 1.2 x 10^6 cells per well. Samples were

incubated with undiluted Human TruStain FcX for 10 min for Fc receptor blocking. Cells were stained for 30 minutes with a mix of antibodies against surface antigens prepared in CyFACS. After the surface stain incubation, samples were washed with CyFACS and fixed/permeabilized with FoxP3 Fixation/Permeabilization Concentrate and Diluent, prepared following manufacturer's guidelines (eBioscience), for 30 minutes. A mix of intracellular antibodies prepared with 1X Perm Wash was added to each sample and incubated for 30 minutes. Cells were washed with 1X Perm Wash and incubated in FoxP3 Fixation/Permeabilization Concentrate and Diluent, prepared following manufacturer's guidelines(eBioscience), containing 191/193Ir DNA Intercalator overnight at 4°C. Prior to fixation, centrifugation steps were performed at 400g at room temperature (23°C) for 5-10 minutes. After fixation, centrifugation steps were performed at 800g at room temperature (23°C) for 5 minutes. All incubations were performed at room temperature unless otherwise specified. Prior to acquisition, samples were transferred to 5 mL round-bottom polystyrene tubes with cell strainer caps, washed and filtered with Cell Staining Buffer (CSB), Cell Acquisition Solution (CAS), and resuspended in CAS supplemented with EQ Four Element Calibration Beads (1:10).

For mass cytometry acquisition, all data was collected on a Helios™ Mass Cytometer (Fluidigm). The instrument was tuned using CyTOF Tuning Solution according to the Helios User Guide (Fluidigm, p. 60-68). A brief overview of tuning steps includes Pre-XY Optimization, Mass Calibration, XY Optimization, DV Calibration, Dual Calibration, Gases/Current Calibration, and QC report. EQ Four Element Calibration Beads (1:10 in CAS) were used according to the manufacturer protocol before and during acquisition. The

data was normalized using the FCS Processing tab of the Fluidigm CyTOF Software 7.0.8493.

Data analysis was manually performed using FlowJo 10.7.1. Samples were initially analyzed by cleaning the data based on the gating strategy previously described.[9] Cell events were gated to remove dead cells and debris through biaxial plots of Time vs. Event Length, Beads (for removal of the EQ Calibration Beads), and Gaussian-derived parameters (Residual, Width, Offset). The OCI-AML3 target cells and NK cells were separated by gating on CD33, CD56 and CD45. The viability stain, 103Rh, was then used to gate out dead cells on both AML and NK cell populations. All viable cells were backgated on both DNA parameters (191Ir and 193Ir) to ensure no doublets were included. NK cell subsets were identified by gating on the $CD14^{-}CD3^{-}CD19^{-}CD33^{-}CD56^{+}$population.

Statistical analysis

For graphs, data are shown as mean ± s.e.m or s.d. as indicated and unless otherwise indicated, statistical differences were evaluated using a two-tailed unpaired Student's *t*-test, assuming equal sample variance. $p < 0.05$ was considered significant. Graphs were produced and statistical analyses were performed using GraphPad Prism 7.0d for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com) or R. P values were two sided and a p value <0.05 was considered statistically significant after any adjustment; *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. Sample size was not specifically

predetermined, but the number of mice used was consistent with prior experience with similar experiments.

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Genentech, Bristol-Myers Squibb, Merck, Novartis, Amgen, Sanofi, Bayer, Pfizer, EMD Serono, Verastem, Aduro, Celldex, and Incyte.

SUPPLEMENTAL FIGURES

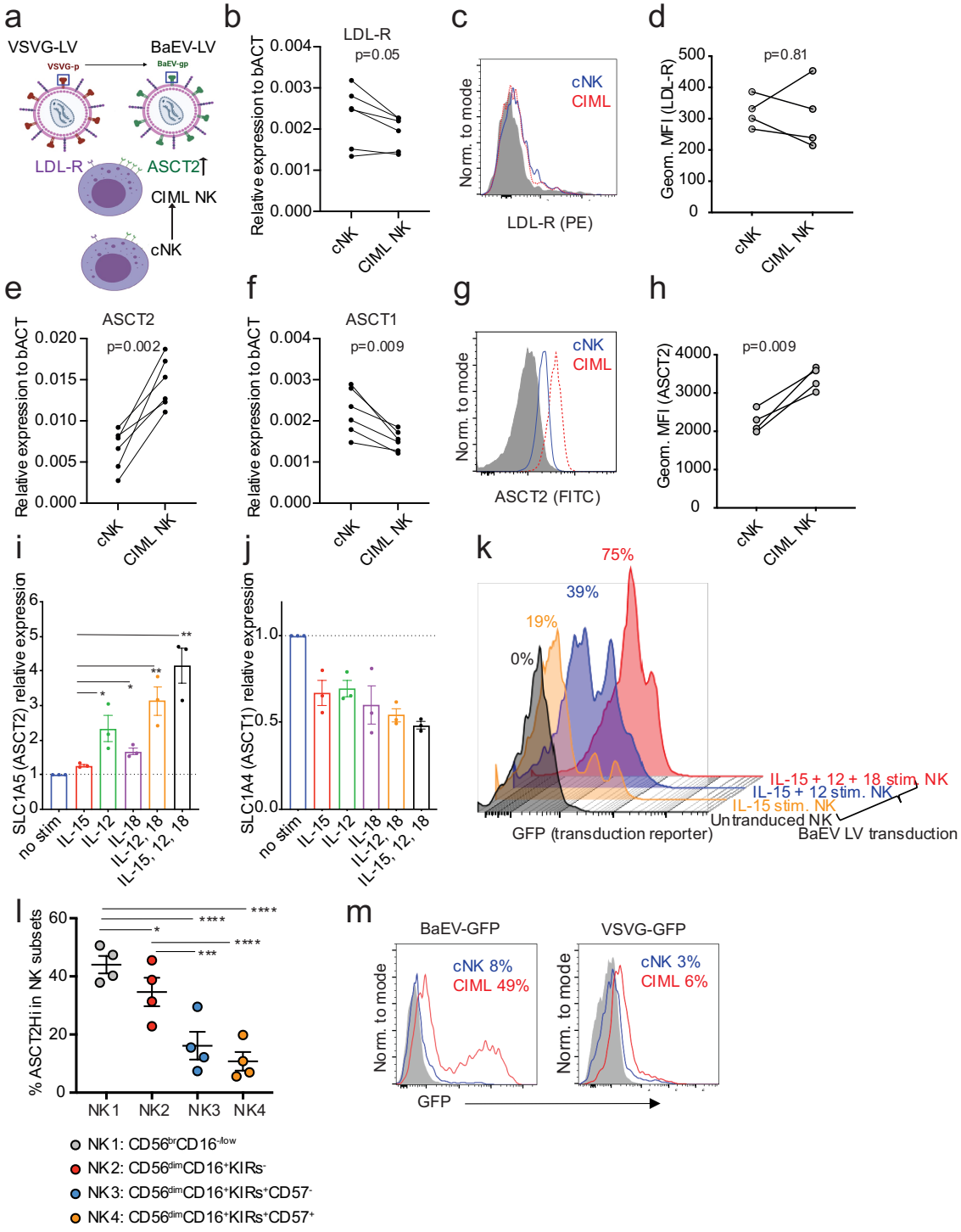

Fig. S1. (Related to Fig. 1) Upregulation of ASCT2 on CIML NK cells promotes their efficient transduction by BaEV-pseudotyped lentiviruses. (a) Schematic diagram

showing LDL-R and ASCT2 expression on primary peripheral blood (PB) derived cNK and CIML NK cells and their transduction by VSVG-LV or BaEV-LV, respectively. (b) RT-PCR quantification of the LDL-R transcript levels in conventional NK (cNK) cells and CIML NK generated from six PB donors. (c) Flow cytometric histograms comparing LDL-R expression in cNK cells (blue histogram) and CIML NK cells (red histogram) from a representative donor. Gray histogram: unstained control. (d) Comparison of geometric mean of LDL-R expression levels in cNK cells and CIML NK cells from four different PB donors. (e-f) RT-PCR quantification of the SLC1A5 (ASCT2) (e) and SLC1A4 (ASCT1) (f) transcript levels in cNK and CIML NK generated from six PB donors. (g) Flow cytometric histograms comparing ASCT2 expression in cNK cells (blue histogram) and CIML NK cells (red histogram) from a representative donor. Gray histogram: unstained control. (h) Comparison of geometric mean of ASCT2 expression levels in cNK cells and CIML NK cells from four different PB donors. (i-k) Requirement of IL-12 and IL-18 in robust upregulation of ASCT2 during CIML differentiation, and corresponding increased transduction efficiency of BaEV lentivirus. Freshly isolated cNK cells from PB were stimulated overnight with indicated cytokine(s) including IL-15 (50 ng/mL) and/or IL-12 (10 ng/mL) and/or IL-18 (50 ng/mL) in RPMI10 medium supplemented with IL-2 (500 U/mL). RT-PCR quantification of the SLC1A5 (ASCT2) (i) and SLC1A4 (ASCT1) (j) transcript levels in cytokine activated NK cells generated from three PB donors. (k) Comparison of BaEV LV-mediated transduction efficiency in NK cells stimulated by IL-12 and/or IL-18 as indicated. Data were representative using one PB donor. (l) Differential expression of ASCT2 in PB NK cell subsets according to their maturation status. Maturation goes up from NK1 to NK4 with indicated markers. (m) Comparison of

transduction efficiency of cNK (blue histogram) and CIML NK (red histogram) cells from the same donor by GFP-expressing lentivirus pseudotyped with BaEV (left panel, MOI=10) and VSVG (right panel, MOI=100). Gray histogram: untransduced control. Each dot in b, d, e, f, h, i, j, l represents one different PB donor. n = 6 in b, e, f; n = 4 in d, h, l; n =3 in i, j. Error bars in i, j, l represented mean with s.e.m.. Data were analyzed by two tailed paired Student's *t*-test (b, d, e, f, h) or one-way ANOVA with the Tukey post-test (l). * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Data are pooled from three (b, d, e, f, h) independent experiments, or representative of three (c, g, k, m) or two (l) independent experiments.

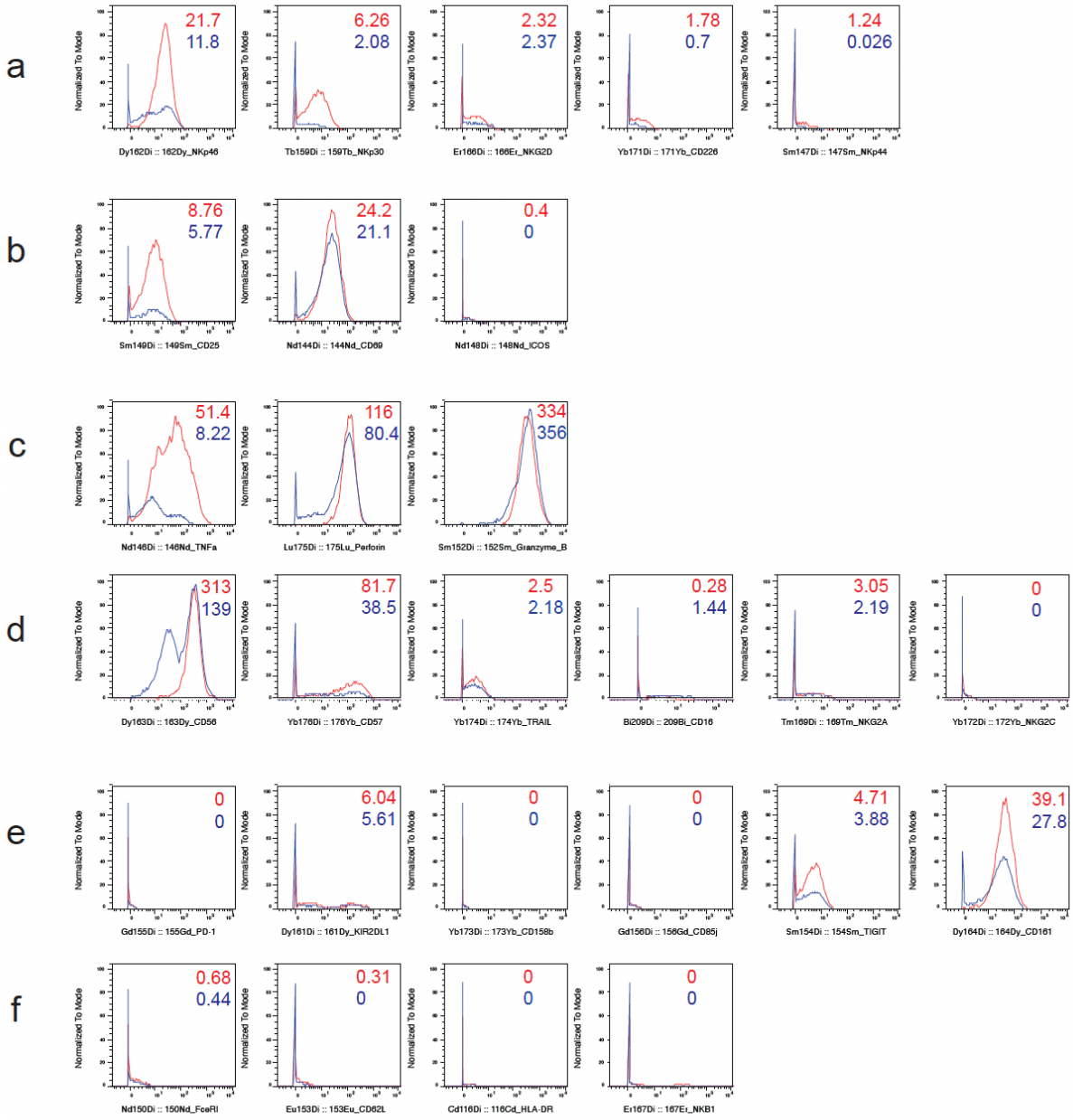

Fig. S2 (Related to Fig. 3a) Phenotypic characterization of CD107a^{+} IFNγ^{+} (shown in red) vs CD107a^{-} IFNγ^{-} (shown in blue) CAR NK cells upon co-culture with OCI-AML3 cells using mass cytometric analysis, median values indicated in the plots. (a) Activating receptors; (b) other activation markers; (c) cytokine and cytotoxicity molecules; (d) maturation markers; (e) inhibitory receptors and surface molecules; (f) other phenotypic markers.

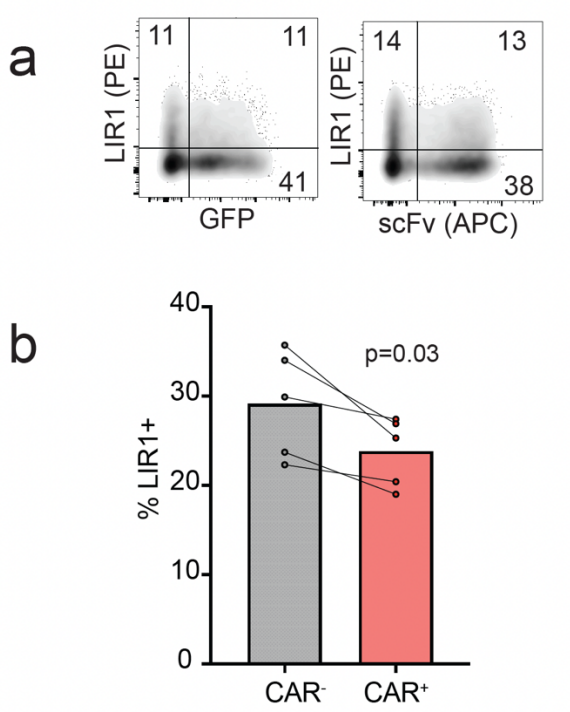

Fig. S3 (Related to Fig. 3) Expression of LIR1 on CAR-transduced CIML NK cells. (a) Representative flow cytometry plots showing correlation between LIR1 expression and CAR transduction with BaEV pseudotyped lentivirus expressing NPM1c-CAR and GFP. (b) Comparison of LIR1 percentages in CAR^- versus CAR^+ CIML NK cells isolated from five PB donors; paired-t test was performed, and bars showed the mean values.

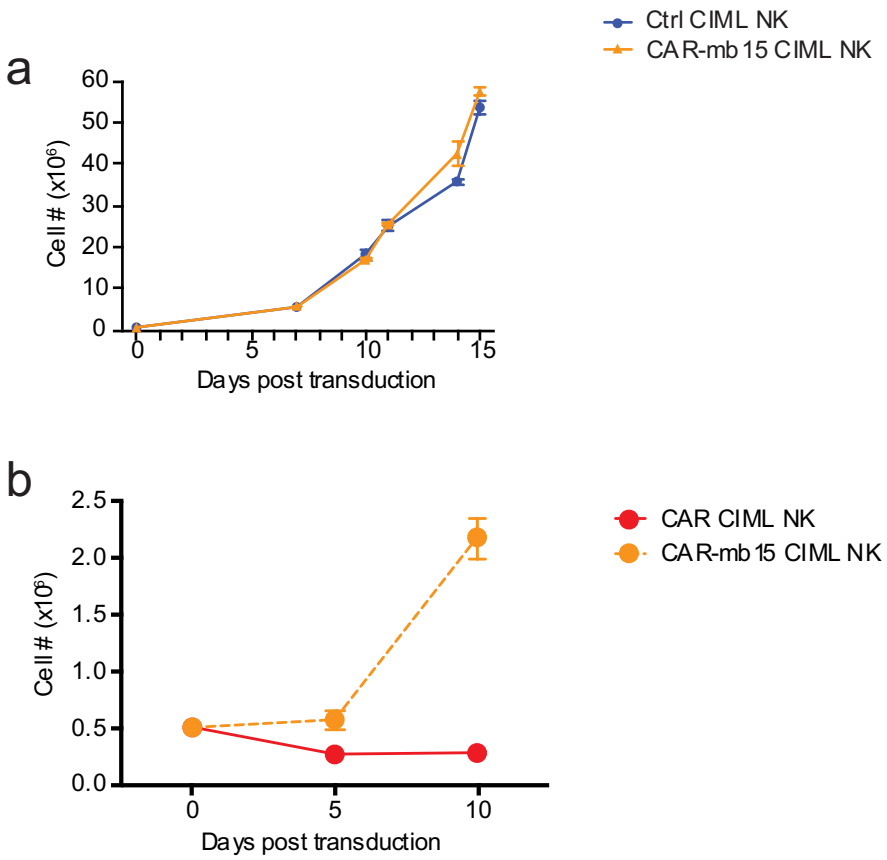

Fig. S4. (Related to Fig. 5). In cytokine-starving conditions, incorporation of mb15 into the CAR significantly improved in vitro expansion of CAR CIML NK cells. (a) When cultured in NK MACS media supplemented with 500 U/ml rhIL-2 and 1ng/ml rIL-15 (with cytokine re-added every 2-3 days), both untransduced (blue) and CAR-mb15-transduced (orange) CIML NK cells can expand vigorously *in vitro*. By day 15 post transduction, there was a 78-fold expansion. (b) Comparison of the absolute cell numbers of CAR (red) versus CAR-mb15 (orange) CIML NK cells at day 5 and day 10 post transduction, under cytokine-starving conditions using NK MACS media without additional cytokine supplementation during the indicated time. Error bars derived from three technical replicates. Data are representative of two (a) or three (b) independent experiments.

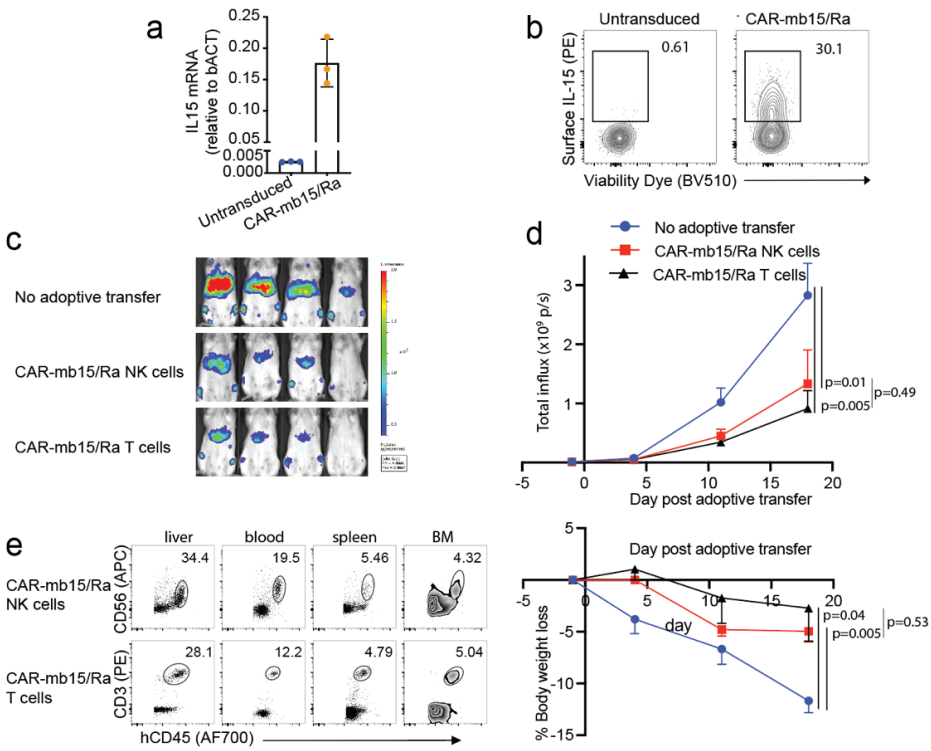

Fig. S5 (Related to Fig. 5) A direct comparison of NPM1c-CAR NK versus CAR T cells in vivo. T cells and CIML NK cells from the same healthy blood donor were transduced with the same CAR-mb15/Ra containing NPM1c CAR and IL-15/IL-15Ra fusion (mb15/Ra). (a) qPCR of *IL15* mRNA expression and (b) flow cytometry analysis of surface staining of IL-15 protein in untransduced or transduced CIML NK cells. (c-e) Equal number of transduced T cells and CIML NK cells ($1x10^6$) were adoptively transferred into OCI-AML3-bearing NSG mice as in Fig. 4. (c) Comparison of tumor burden in recipient mice without any adoptive cell transfer, CAR-transduced CIML NK cells or T cells by BLI at day 11 post immune cell infusion and (d) quantification of luciferase activity and body weight during the time course (n=4 mice per group). (e) Endpoint flow cytometry analyses showing persistence of the transferred human NK and T cells in the indicated tissues of NSG recipient mice at day 19 post adoptive transfer. The numbers indicate percentages of cells in the gated regions.

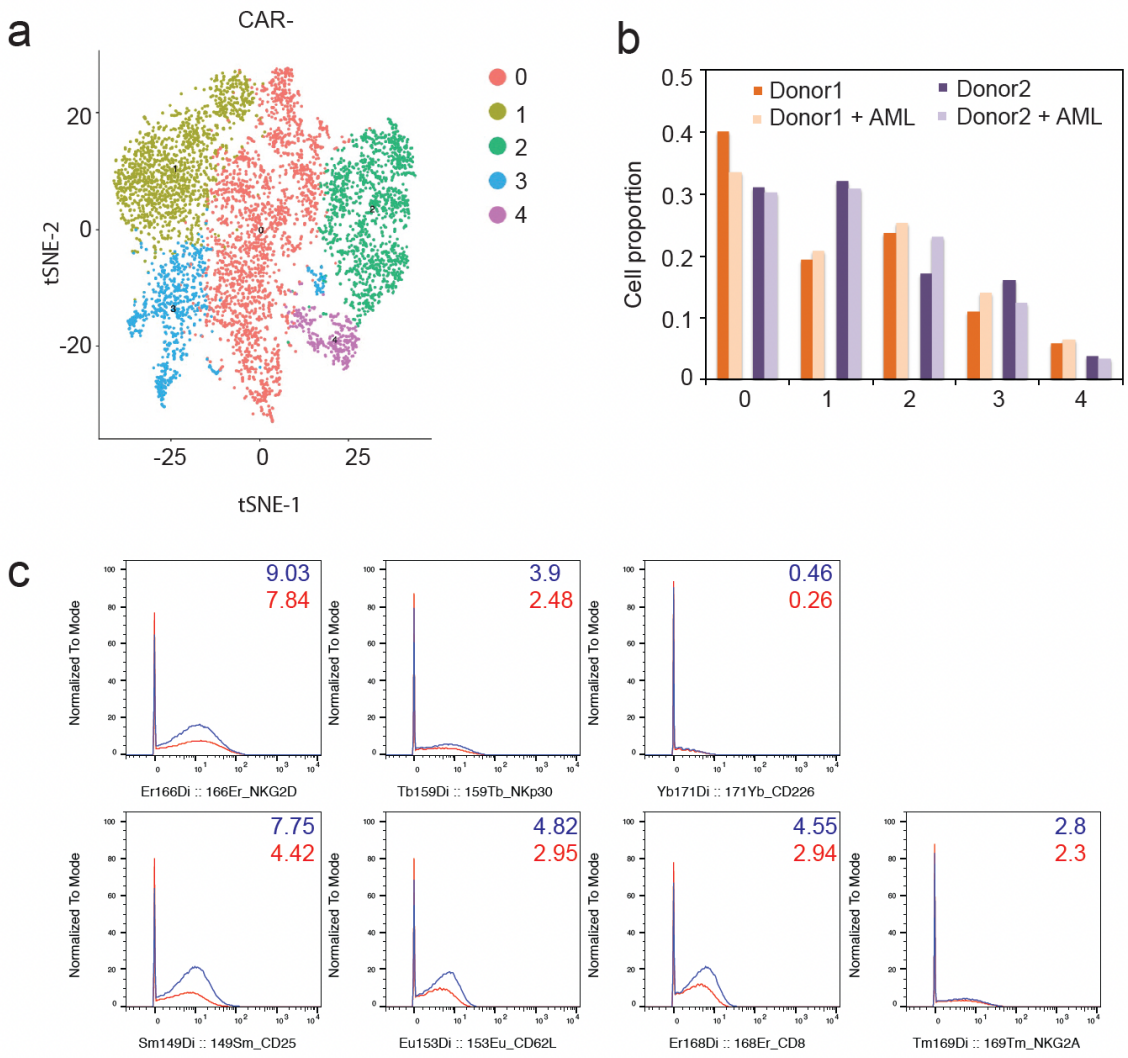

Fig. S6. (Related to Fig. 6). scRNA-seq analyses of CAR$^-$ CIML NK cells in responses to OCI-AML3 target cells. Within the same experiment shown in Fig. 6a and b, CIML NK cells generated from two PB donors were transduced with NPM1c-CAR-mb15 lentivirus, and purified viable NK cells were co-cultured with OCI-AML3 cells for 24 hrs followed by scRNA-seq. (a) tSNE clustering analysis of CAR$^-$ NK cells co-cultured with or without AML target cells. (b) Cell proportion of identified clusters in CAR$^-$ NK cells co-cultured with or without AML target cells. (c) Mass cytometric analyses in Fig. 6h before data normalization.

Table S1. Taqman probe for qPCR.

Target gene	Assay number	Lot number
IL15	Hs01003716_m1 IL15	lot # 1837025
LDLR	Hs00181192_m1 LDLR FAM	lot # P180429-001 B03
SLC1A4	Hs00983079_m1 SLC1A4 FAM	lot # P200602-004 B01
SLC1A5	Hs01056542_m1 SLC1A5 FAM	lot #P200602-004 B02
ACTB	Hs01060665_g1 ACTB	lot # 1637218

Table S2. Mass cytometry panel antibodies for NK cell phenotyping and functional assessment.

Antibodies	Vender	Cat. no.
Anti-Human CD45-89Y	Fluidigm	3089003B
Purified anti-human HLA-DR (Maxpar® Ready) Antibody	Biolegend	307651
Anti-Human CD3-141Pr	Fluidigm	3141019B
Anti-Human CD19-142Nd	Fluidigm	3142001B
Anti-Human CD127[IL-7Ra]-143Nd	Fluidigm	3143012B
Anti-Human CD69-144Nd	Fluidigm	3144018B
Anti-Human CD4-145Nd	Fluidigm	3145001B
Anti-Human TNFα-146Nd	Fluidigm	3146010B
Ultra-LEAFTM Purified anti-human CD336 (NKp44) Antibody	Biolegend	325121
Anti-CD278/ICOS-148Nd	Fluidigm	3148019B
Anti-Human CD25-149Sm	Fluidigm	3149010B
Anti-Human FceRI-150Nd	Fluidigm	3150027B
Anti-Human CD107a-151Eu	Fluidigm	3151002B
Granzyme B Antibody	Novus Bio	NBP1-50071
Anti-Human CD62L-153Eu	Fluidigm	3153004B
Anti-Human TIGIT-154Sm	Fluidigm	3154016B
Anti-Human CD279/PD-1-155Gd	Fluidigm	3155009B
Anti-Human CD85j-156Gd	Fluidigm	3156020B
Anti-Human CD33-158Gd	Fluidigm	3158001B

Anti-Human CD337/NKp30-159Tb	Fluidigm	3159017B
Anti-Human CD14-160Gd	Fluidigm	3160001B
KIR2DL1/CD158a Antibody	Novus Bio	NBP2-11758
Anti-Human CD335/NKp46-162Dy	Fluidigm	3162021B
Anti-Human CD56-163Dy	Fluidigm	3163007B
Anti-Human CD161-164Dy	Fluidigm	3164009B
Anti-Human IFNγ-165Ho	Fluidigm	3165002B
Anti-Human CD314/NKG2D-166Er	Fluidigm	3166016B
Anti-Human CD158e1/NKB1-167Er	Fluidigm	3167013B
Anti-Mouse CD8a-168Er	Fluidigm	3168003B
Anti-Human CD159a/NKG2A-169Tm	Fluidigm	3169013B
Anti-Human CD226-171Yb	Fluidigm	3171013B
Human NKG2C/CD159c Antibody	R&D	MAB1381
Anti-Human CD158b-173Yb	Fluidigm	3173010B
TRAILR4/TNFRSF10D/DcR2 Antibody - Azide Free	Novus Bio	NBP1-45027
Anti-Human Perforin-175Lu	Fluidigm	3175004B
Anti-Human CD57-176Yb	Fluidigm	3176019B
Anti-Human CD16-209Bi	Fluidigm	3209002B