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CAR-T cells targeting a nucleophosmin neoepitope exhibit potent specific activity in mouse models of acute myeloid leukaemia

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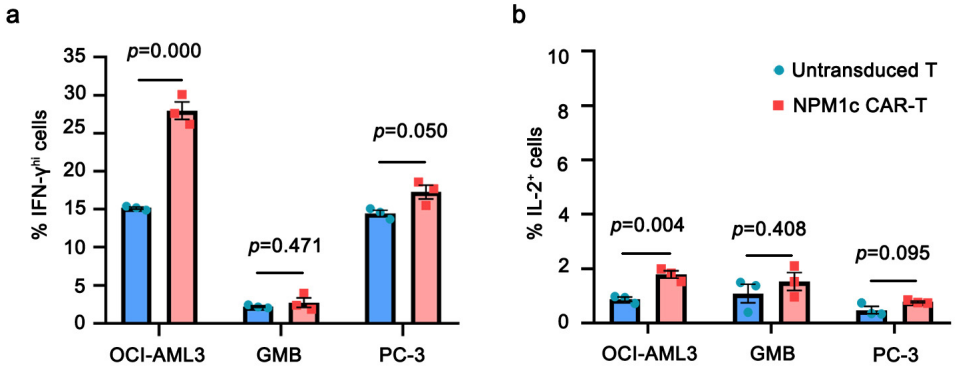
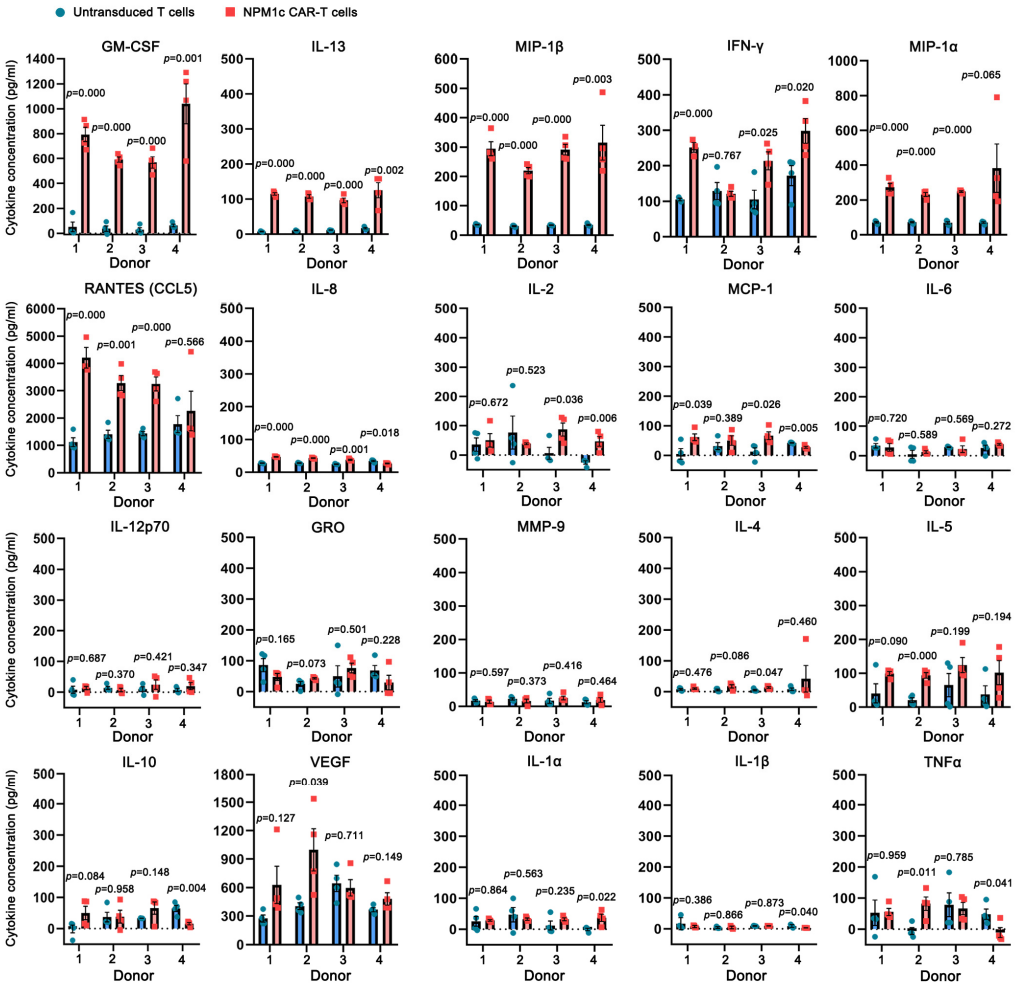
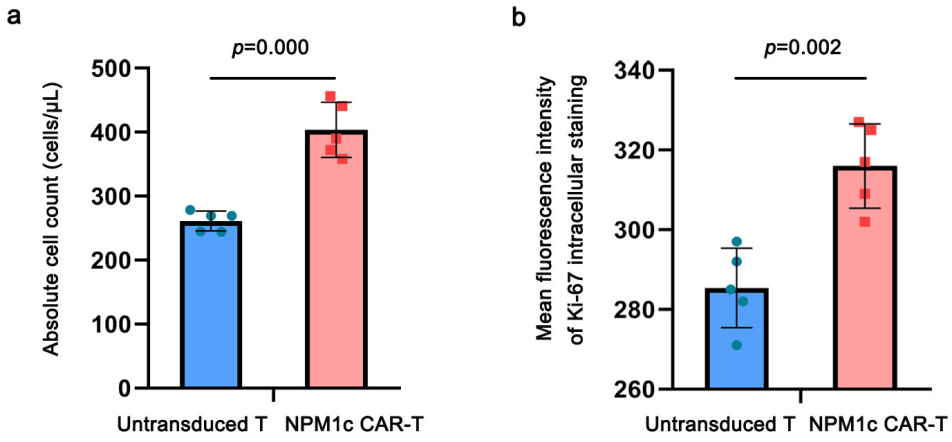
Supplementary Figures



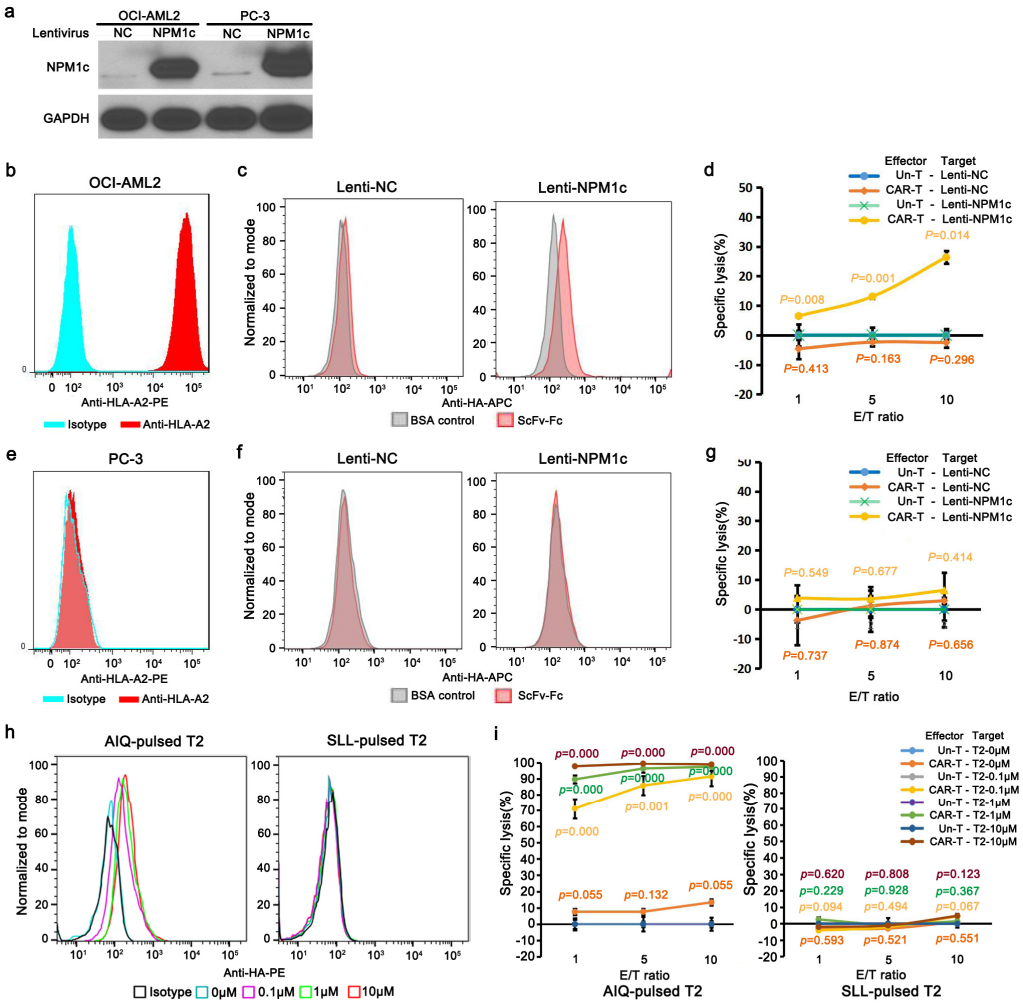
Supplementary Fig. 1 | Comparison of cytokine expression between CAR-T cells and untransduced T cells. a, NPM1c CAR-T cells and untransduced T cells were co-cultured with OCI-AML3, GMB or PC-3 in the presence of monensin and brefeldin A for 12 hrs. Cells were stained for CD3 and then permeabilized and stained for intracellular IFN-©, followed by flow cytometry. Percentages of IFN-©$^{+}$ NPM1c CAR-T cells and untransduced T cells are shown. b, Percentages of IL-2^{+} NPM1c CAR-T cells and untransduced T cells are shown. Graphing was performed using GraphPad Prism v8.00 software; n = 3 biologically independent samples; graph bars and error bars represent the mean ± s.e.; *p* values (two-sided independent samples t-test) are indicated.



Supplementary Fig. 2 | NMP1c CAR-T cells are stimulated to secrete multiple cytokines by NPM1c$^+$HLA-A2$^+$ target cells. NPM1c CAR-T cells or untransduced T cells were co-cultured with NPM1c$^+$HLA-A2$^+$ OCI-AML3 cells for 16 hours. Culture supernatants were collected and assayed for 20 different cytokines simultaneously using an Quantibody Human Cytokine Array. Each cytokine contained quadruplicate antibody spots. T cells from 4 different healthy donors were separately analyzed. Concentration of each cytokine was calculated by ELISA Calc software. Graphing was performed using GraphPad Prism v8.00 software; unless otherwise specified, all of cytokines contained 4 replicate antibody spots (n = 4); 3 replicate spots for IFN-γ from donor 1; 3 replicate spots for RANTES of untransduced T cells from donor 1; graph bars and error bars represent the mean ± s.e.; *p* values (two-sided independent samples t-test) are indicated.

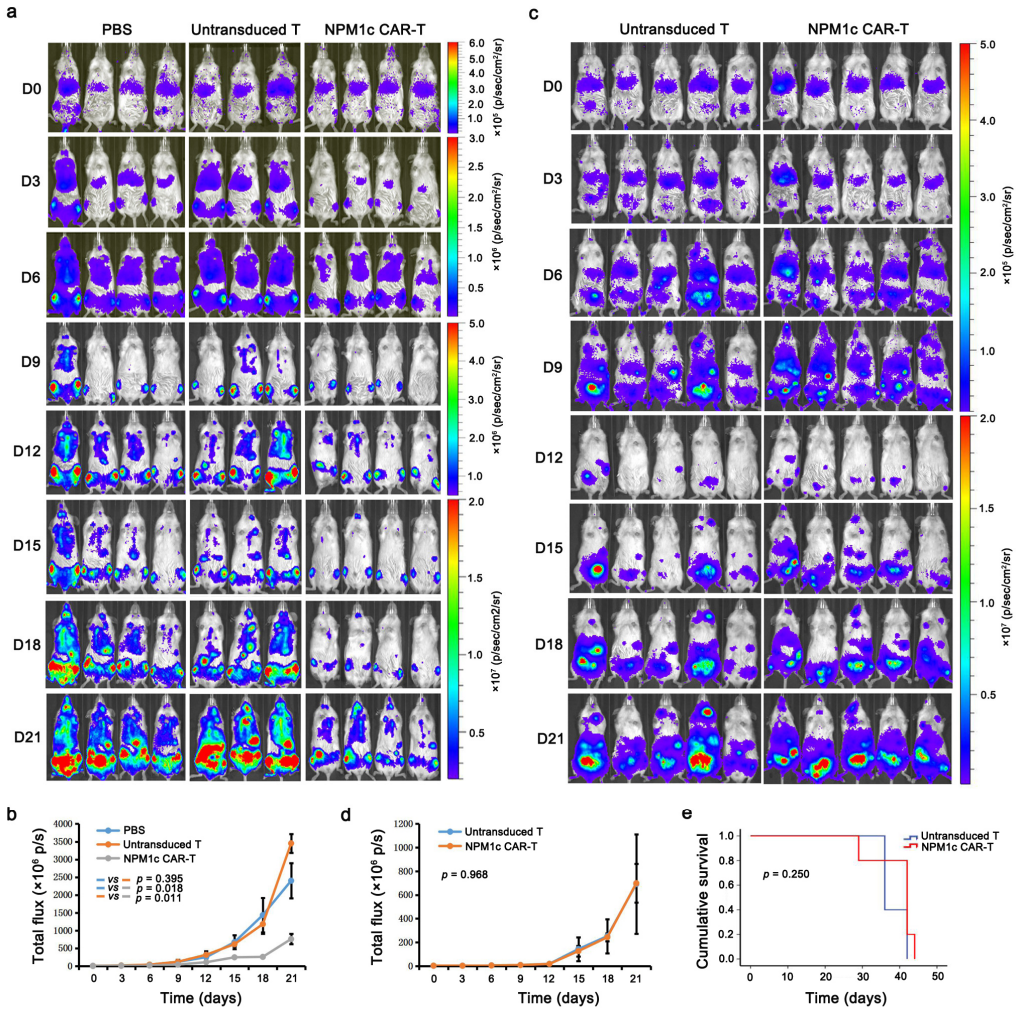


Supplementary Fig. 3 | NPM1c CAR-T cells proliferate in response to NPM1c^{+}HLA-A2^{+} target cells. NPM1c CAR-T cells or untransduced T cells were co-cultured with OCI-AML3 cells for 5 days. The absolute cell number of CAR-T cells or untransduced T cells was determined by flow cytometry using precision count beads. Ki-67 expression by NPM1c CAR-T cells or untransduced T cells were assayed by intracellular staining followed by flow cytometry. a, Comparison of the numbers of NPM1c CAR-T cells and untransduced T cells at day 5. b, Comparison of mean fluorescence intensity (MFI) of intracellular Ki-67 staining between untransduced T cells and NPM1c CAR-T cells. Graphing for a and b was performed using GraphPad Prism v8.00 software; the *p* values (two-sided independent samples t-test) indicate comparison between NPM1c CAR-T cells and untransduced T cells; n = 5 biologically independent samples; graph bars and error bars represent the mean ± s.e..



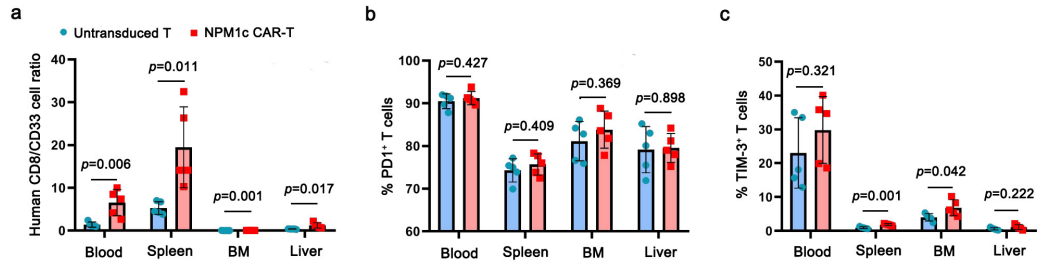
Supplementary Fig. 4 | NPM1c CAR-T cells specifically kill HLA-A2$^+$ NPM1c$^+$ human tumor cells in vitro. a-g, OCI-AML2 and PC-3 cells were transduced with lentivirus expressing NPM1c (Lenti-NPM1c) or just empty negative control (Lenti-NC). Transduced cells were sorted and expanded for various experiments. a, Western blotting analysis of cell lysates of the transduced cells with antibody specific for the mutant NPM1c. Representative data from three separate experiments. GAPDH was used as loading control. The images of the full scan with the boundaries of the crops outlined were provided in The full scans of the cropped gels and blots. b and e, Flow cytometry analysis of HLA-A2 expression by OCI-AML2 cells (b) and PC-3 cells (e). Red histograms: stained with anti-HLA-A2, and cyan histograms: stained with isotype control antibody. Representative data from technical triplicates is shown. c and f, Flow cytometry analysis of AIQ-HLA-A2 expression by OCI-AML2 cells (c) and PC-3 cells (f) transduced with control Lenti-NC (left) or lenti-NPM1c (right). Red histograms: stained with YG1 scFv-Fc and anti-HA, and gray histograms: stained with BSA followed by anti-HA. Representative data from three separate experiments with technical triplicates is shown. d and g, Comparison of specific killing of transduced OCI-AML2 cells (d) and PC-3 cells (g) by NPM1c CAR-T cells or untransduced T cells. NPM1c CAR-T cells or untransduced T cells were co-cultured with transduced

OCI-AML2 or PC-3 target cells at the indicated E:T ratios for 24 hours. Target cell killing was measured by assaying the luciferase activity of the surviving target cells. The percentages of specific lysis of tumor cells at different E:T ratios were calculated (see Supplemental Materials and Methods). Target cells and T cells in each reaction are shown. h, Flow cytometry analysis of YG1 scFv-FC binding to T2 cells pulsed with different concentrations of AIQ peptide (left) or SLL peptide (right). Representative data from three separate experiments. i, Comparison of specific killing of T2 cells pulsed with different concentrations of AIQ (left) or SLL (right) peptide by NPM1c CAR-T cells or untransduced T cells. NPM1c CAR-T cells or untransduced T cells were co-cultured with peptide-pulsed T2 target cells at the indicated E:T ratios for 24 hours. Target cell killing was measured by assaying the luciferase activity of the surviving target cells. Graphing for d, g and i was performed using Microsoft Office 2016; the *p* values (two-sided independent samples t-test) indicate comparison between NPM1c CAR-T cells and untransduced T cells at the same E:T ratio; n = 3 biologically independent samples; data points and error bars represent the mean ± s.e.

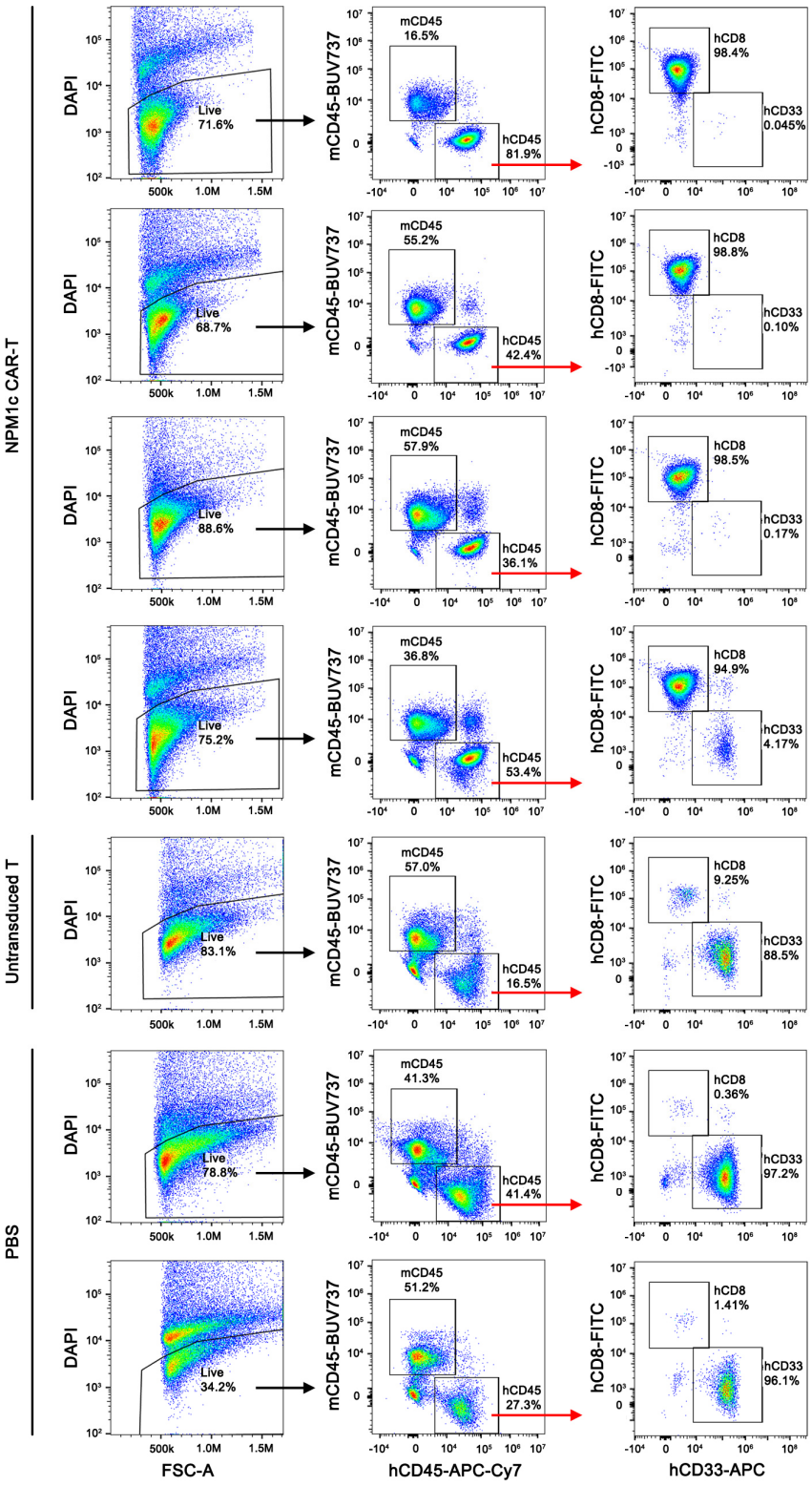


Supplementary Fig. 5 | NPM1c CAR-T cells kill NPM1c-positive AML cells but not NPM1c-negative AML cells. a, Comparison of OCI-AML3 leukemia burden by bioluminescence imaging among mice given NPM1c CAR-T cells, untransduced T cells or PBS at the indicated days (D0 to D21) post T cell/PBS injection (n=3-4). b, Comparison of the total flux (luciferase signals from systemic OCI-AML3 cells) in mice (from a) treated with NPM1c CAR-T cells (n=4), untransduced T cells (n=3), or PBS (n=4). c, Comparison of OCI-AML2 leukemia burden by bioluminescence imaging between mice treated with NPM1c CAR-T cells (n=5) and untransduced T cells (n=5) at the indicated days (D0 to D21) post T cell injection. d, Comparison of the total flux (luciferase signals from systemic OCI-AML2 cells) in mice from c. e, Kaplan Meier survival curves of mice treated with either NPM1c CAR-T cells (n=5) or untransduced T cells (n=5) from c. The scales for imaging are shown at the right of a and c. For better visualization, different scales were used at day 0, day 3-6, day 9-12 and day 15-21 for a, and day 0-9 and day 12-21 for c. Graphing for total flux was performed using Microsoft Office 2016, and graphing for survival curves was performed using SPSS Statistics 22 software; data points and error bars represent the mean ± s.e.; *p* value (two-way repeated-measures ANOVA for total flux and two-sided

Mantel-Cox log-rank test for survival comparison) is indicated.

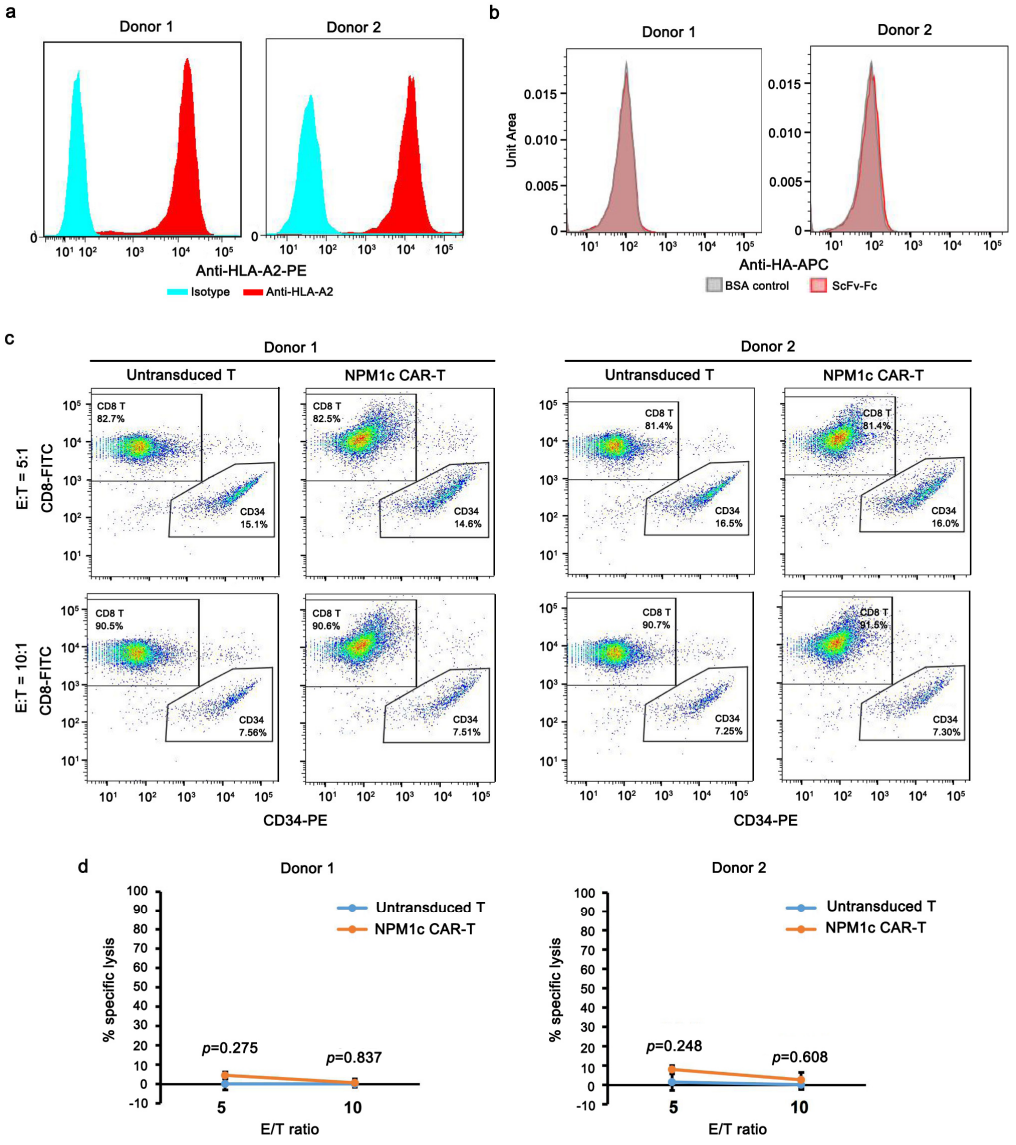


Supplementary Fig. 6 | NPM1c CAR-T cells reduce leukemia burden in blood, spleen, bone marrow and liver. a, Ratios of percentages of hCD8$^+$ T cells over hCD33$^+$ leukemic cells in different tissues in mice given NPM1c CAR-T cells or untransduced T cells. b and c, The percentages of PD1$^+$ T cells (b) or Tim-3$^+$ T cells (c) among human CD8$^+$ T cells in different tissues. Blue bars represent treatment with untransduced T cells, pink bars represent treatment with CAR-T cells. Graphing was performed using GraphPad Prism v8.00 software; graph bars and error bars represent the mean ± s.e.; *p* values (two-sided independent samples t-test) are indicated (n=5).



Supplementary Fig. 7 | NPM1c CAR-T cells effectively eliminate leukemia cells in the bone marrow 30 days post CAR-T cell injection. NSG mice were engrafted with OCI-AML3 and 4 days later injected with NPM1c CAR-T cells, untransduced T cells or PBS (mice were shown in Supplemental Fig. 5a). 30 days after T cell injection, cells were harvested from the bone marrow of surviving mice. Cells were stained for mouse

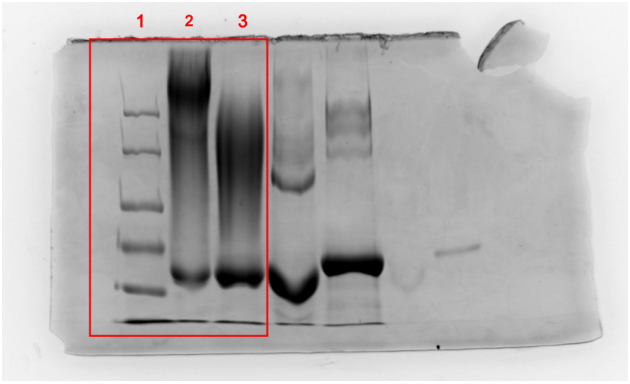
CD45 and human CD45, CD8, and CD33, followed by flow cytometry. Shown are FSC versus DAPI staining profiles of total cells (left), hCD45 vs mCD45 staining profiles gaining on live (DAPI$^-$) cells (middle), and hCD33 vs hCD8 staining profiles of hCD45$^+$ cells (right). The numbers indicate percentages of cells in the gated regions. The experiment was repeated twice independently with similar results.



Supplementary Fig. 8 | NPM1c CAR-T cells do not exhibit cytotoxicity towards normal human HLA-A2$^+$ CD34$^+$ hematopoietic stem/progenitor cells (HSPCs). Human CD34$^+$ HSPCs were purified from two donor fetal livers using EasySep Human CD34 Positive Selection Kit. a, Flow cytometry analysis of HLA-A2 expression by HSPCs. Red histograms: stained with anti-HLA-A2, and cyan histograms: stained with isotype control antibody. Representative data from technical triplicates are shown. b, Flow cytometry analysis of HSPCs for YG1 scFv-Fc binding. Red histograms: stained with YG1 scFv-Fc and anti-HA, and gray

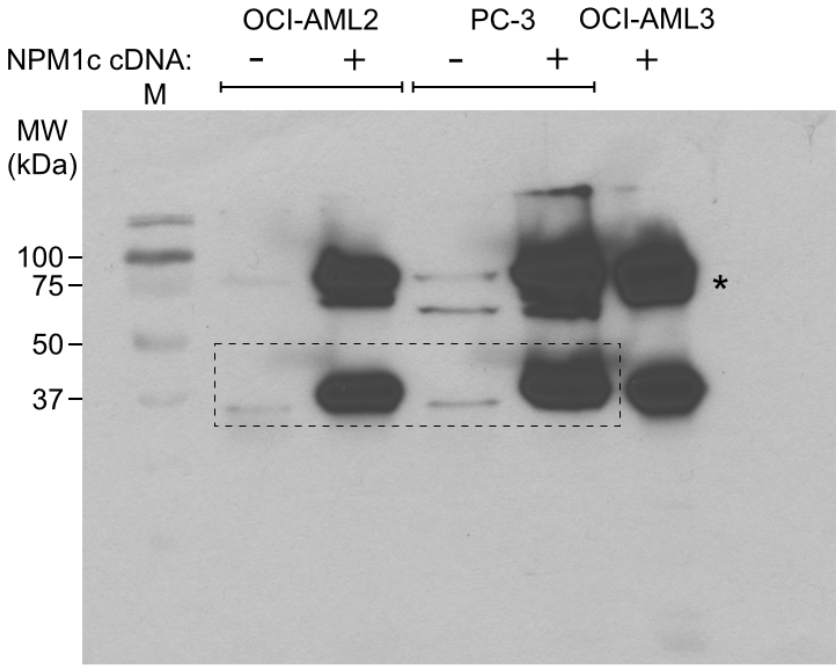
histograms: stained with BSA followed by anti-HA. Representative data from three separate experiments with technical triplicates are shown. c, NPM1c CAR-T cells do not kill HLA-A2$^+$ CD34$^+$ HSPCs. NPM1c CAR-T cells and untransduced T cells were incubated with HSPCs at the indicated E:T ratios for 24 hours. The cell mixtures were stained for CD8 plus CD34 and quantified by flow cytometry with precision count beads. Shown are examples of CD8 (T cells) versus CD34 (HSPCs) staining profiles at different E:T ratios. The percentages of cells in the gated regions are indicated. d, Comparison of specific lysis at different E:T ratios between NMP1c CAR-T cells and untransduced T cells (see Supplemental Materials and Methods). Graphing for d was performed using Microsoft Office 2016; the *p* values (two-sided independent samples t-test) indicate comparison between NPM1c CAR-T cells and untransduced T cells at the same E:T ratio; n = 3 biological replicates; data points and error bars represent the mean ± s.e..

The full scans of the cropped gels and blots



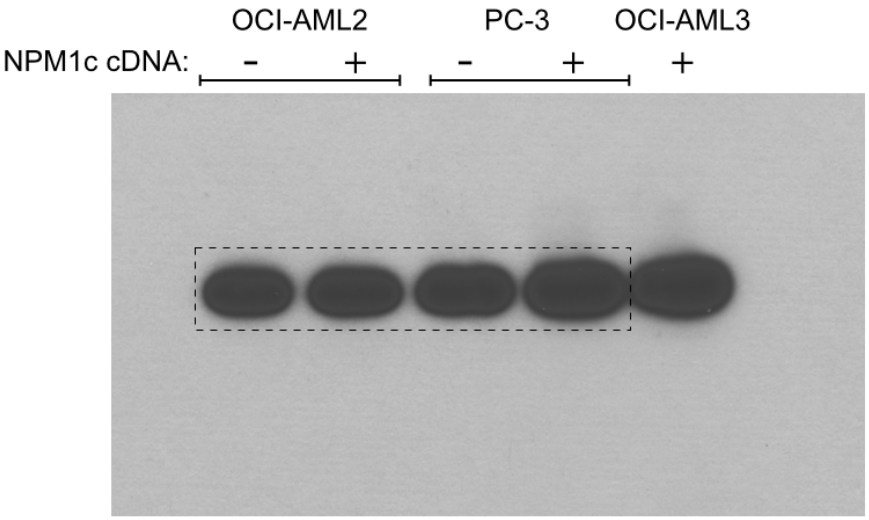
Full scans of Fig. 2b | Un-scropped SDS-PAGE gels related to Fig. 2b. Lane 1: protein ladder, lane 2: scFv-Fc protein (1µg), nonreduced, lane 3: scFv-Fc protein, reduced (1µg).

TOP: α-NPM1c (mutant epitope)



* = Un-cleaved NPM1c-P2A-mCherry (predicted MW~80 kDa)

BOTTOM: α-GAPDH



Full scans of Supplementary Fig. 4a | Un-scropped Western blots related to Supplementary Fig. 4a.

Supplementary Methods

Cytotoxicity assay by measuring luciferase activity

Cytotoxicity assays of CAR-T cells were performed using luciferase-expressing target cell lines. T cells were incubated with target cells at indicated effector:target (E:T) ratios for 24 hr. Cells were then rinsed once in PBS, lysed in luciferase cell culture lysis reagent (Promega), and subsequently mixed with luciferase assay reagent (Promega). Luminescence of the lysates was analyzed using a plate spectrophotometer (Infinite M200PRO, TECAN). The luminescence of target cells alone was used as a baseline control. Specific lysis of each sample was calculated using the following formula: specific lysis (%) = 100 × {1 – [(luminescence in CAR-T group/luminescence in target cells alone)/(luminescence in untransduced T group/luminescence in target cells alone)] }.

Quantibody Human Cytokines Array

Quantibody Human Cytokines Array1 (QAH-CYT-1) was purchased from Raybiotech. One glass slide is spotted with 16 wells of identical cytokine antibody arrays. Each well contained quadruplicate antibody spots for 20 human cytokines. 2×10^5 of NPM1c CAR-T cells or untransduced T cells that were derived from 4 healthy donors were co-cultured with 1×10^5 of $NPM1c^+HLA\text{-}A2^+$ OCI-AML3 cells in RPMI-1640 containing 10% FBS without any cytokines in 96-well round bottom plates at 37 °C, 5% CO_2 for 16 hours, and then 100 µl of cell-free supernatants were collected from each well for cytokine quantification. The array slides were treated and processed according to manufacturer instructions. Some of the slide wells were treated with pure antigens in order to calculate a standard curve. Fluorescence signals were detected by a laser scanner (Axon GenePix). Data extraction was done using the GAL file (www.RayBiotech.com/Gal-Files.html.) that is specific for this array along with the microarray analysis software (GenePix), and F488 total intensity from spots was analyzed by ELISA Calc software. A standard curve of known concentration of each cytokine was established, and the concentration of cytokine in the samples was then calculated by interpolation onto the standard curve.

Cytotoxicity assay by flow cytometry with precision count beads

Patient-derived primary AML samples were purchased from the Dana-Farber Cancer Institute. NPM1c CAR-T cells were incubated with primary AML cells at indicated effector:target ratios. After 24-hrs incubation, cells were washed in PBS without serum and stained using Live/Dead Fixable Dead Cell Stain Kits (Invitrogen) according to the manufacturer's protocol. Cells were then stained with FITC-anti-human CD8, PE-anti-human CD33 on ice for 30 min in the dark. Cells were washed with FACS buffer, resuspended with 180 µl FACS

buffer and then 20 µl of precision count beads (BioLegend) were added into cell suspension according to manufacturer instructions, in a total volume of 200 µl. Cells were analyzed by flow cytometry (LSRII HTS-1). Absolute cell count of primary AML cells was calculated according to the formula in manufacturer instructions. Specific lysis of each sample was calculated using the following formula: specific lysis (%) =100 × [1 – (absolute cell count of primary AML cells in CAR-T group /absolute cell count of primary AML cells in untransduced T group)].

Human HLA-A2$^+$ CD34$^+$ hematopoietic stem/progenitor cells (HSPCs) were purified from fetal livers of two donors by EasySep Human CD34 Positive Selection Kit (StemCell Technologies) as previously described[1, 2]. NPM1c CAR-T cells were incubated with CD34$^+$ HSPCs at indicated effector:target ratios. After 24-hrs incubation, cells were washed in PBS without serum and stained using Live/Dead Fixable Dead Cell Stain Kits (Invitrogen) according to the manufacturer's protocol. Cells were then stained with FITC-anti-human CD8, PE-anti human CD34 on ice for 30 min in the dark. As described above, cells were washed with FACS buffer, resuspended with 180 µl FACS buffer and then 20 µl of precision count beads (BioLegend) were added into cell suspension according to manufacturer instructions, in a total volume of 200 µl. Cells were analyzed by flow cytometry (LSRII HTS-1). Absolute cell count of CD34$^+$ HSPCs was calculated according to the formula in manufacturer instructions. Specific lysis of each sample was calculated using the following formula: specific lysis (%) =100 × [1 – (absolute cell count of CD34$^+$ HSPCs in CAR-T group /absolute cell count of CD34$^+$ HSPCs in untransduced T group)]. The use of human tissue in this study was approved by the Institutional Review Board at Massachusetts Institute of Technology.

CAR-T cell killing of primary HLA-A2$^+$ NPM1c$^+$ AML xenografts

Patient-derived primary NPM1c$^+$ HLA-A2$^+$ AML samples were purchased from the Dana-Farber Cancer Institute. The NSG-SGM3 (NSGS) mice with 10-week age were purchased from the Jackson Laboratories and housed in the specific pathogen-free (SPF) vivarium at the Massachusetts Institute of Technology (MIT). To improve engraftment efficiency of human primary AML in mice, NSGS mice were hydrodynamically injected with 100 µg DNA plasmids encoding human IL-3 and GM-CSF 24 hours before primary AML injection, as previously described[3]. NSGS mice were irradiated at 250 cGy, followed by tail vein injection with 1×10^6 of primary NPM1c$^+$ HLA-A2$^+$ AML cells within 24 hours post irradiation. After 2 weeks, 1×10^7 CAR-T cells that were sorted based on GFP expression, or the same number of activated but untransduced human T cells were injected into the leukemia-bearing mice. AML burden was quantified in the peripheral blood by tail vein bleeding and analyzed by flow cytometry (LSR Fortessa HTS-2, BD Biosciences) every 9 days. AML

engraftment was defined as the percentage of circulating human $CD45^+$ $CD8^-$ cells.

Proliferation assays

NPM1c CAR-T cells or untransduced T cells (1×10^5) were co-cultured with 1×10^5 of $NPM1c^+HLA\text{-}A2^+$ OCI-AML3 cells in RPMI-1640 containing 10% FBS without any cytokines in 96-well round bottom plates at 37 °C, 5% CO_2 for 5 days. Cells were then harvested, stained with Live/Dead Fixable Aqua Dead Cell Stain (Invitrogen), FITC-anti-human CD8 (BioLegend), and PE-anti-human CD33 (BioLegend), and precision count beads (BioLegend) were added prior to flow cytometry analysis. Absolute cell count of $CD8^+$ T cells was calculated according to the formula in manufacturer instructions of precision count beads. For intracellular Ki-67 staining, cells were washed, stained with Live/Dead Fixable Aqua Dead Cell Stain, followed by surface staining for CD8, then fixed, permeabilized, and intracellularly stained with PE-anti-human Ki67 (BioLegend) according to the manufacturer's protocol. Cells were analyzed by flow cytometry (LSRII HTS-1) and live $CD8^+$ lymphocytes were gated for further analysis.

Western blotting

Equal number of cells were washed with PBS and lysed in RIPA buffer supplemented with protease inhibitors. Total cell extracts were dissolved in SDS Loading Buffer, boiled for 5 minutes at 95°C, separated using 10% SDS-PAGE, and transferred to a PVDF membrane. After blocking the membrane with 5% non-fat milk TBS-T solution, the membrane was probed with antibodies specific for the mutant NPM1c (NB110-61646SS; NovusBio) or GAPDH (#3683; Cell Signaling Technology).

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