Supplementary Information

Off-the Shelf-CAR-Engineered Natural Killer Cells Targeting FLT3 Enhance Killing of Acute Myeloid Leukemia

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Running title: FLT3-CAR NK Cells Targeting AML

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Supplementary Methods

Generation of FLT3-specific CAR lentivirus and retrovirus.

Codon optimized soluble IL-15 (s15), membrane-bound IL-15 (m15), and soluble IL-15/IL-15Rα (s15c) were directly amplified from the DNA templates provided by CytoImmune Therapeutics, Inc. and inserted into a clinical-grade retroviral vector (RRV) at the SalI and MluI sites. We constructed and expressed three different forms of IL-15 in NK cells: soluble IL-15 (sIL15) (UniProtKB Primary accession: P40933), soluble IL-15/IL-15Rα (sIL15c) (15Rα UniProtKB Primary accession: Q13261), and membrane-bound IL-15 (mIL15) with a luciferase enzyme. The transmembrane domain of mIL15 is derived from PDGFR (UniProtKB Primary accession: P09619). tCD19 NK cells expressing a luciferase enzyme were used as control. The FLT3-CAR expressed in NK-92 cells was generated using the method that we previously reported[1]. For the FLT3-CAR vector with IgG1 as the hinge region used for retroviral transduction of umbilical cord blood (UCB) NK cells, the retroviral construct encoded the codon-optimized anti-FLT3 single-chain fragment variable (scFv) along with either CD28-CD3ζ, 2B4-CD3ζ, or CD28-CD3ζ and s15-P2A-tEGFR domains provided by CytoImmune Therapeutics, Inc.

Isolation of UCB NK cells and generation of FLT3 CAR_s15 NK cells

NK cells from UCB were isolated by using the human NK cell enrichment cocktail and Ficoll-Paque, followed by cryopreservation. Frozen UCB NK cells were thawed, expanded, and activated with irradiated K562 feeder cells expressing membrane-bound IL-21 and 4-1BBL (APC K562) in the presence of recombinant human IL-2. Expanded NK cells were transduced with retroviral constructs expressing FLT3 CAR_s15 or control vectors on day 5 in RetroNectin-coated plates, according to the manufacturer's protocol. The engineered NK cells were further expanded and

activated by co-culturing the cells with irradiated APC K562 cells for additional days prior to being harvested for freezing in liquid nitrogen for subsequent in vitro and in vivo studies.

Enzyme-linked immunosorbent assay (ELISA)

Measurement of the production of IFN-γ by NK cells was measured as previously described in our published protocol.[2] Soluble IL-15 was measured using the human IL-15 Quantikine ELISA kit (R&D). The IL-15/ILRα complex was measured using the human IL-15/IL-15R alpha Complex DuoSet ELISA (R&D).

Cytotoxicity assay

A standard 4-hour ^{51}Cr release assay was performed to measure the capacity of NK cells to lyse AML cells as described previously.[2] Flow cytometry-based cytotoxicity assay (Agilent Technologies) was performed by co-culturing NK or CAR NK cells with the target cells expressing ZsGreen in 96-well plates at multiple E:T ratios in 200 µl of media, followed by data collection after 48h of co-culture. The gate was set to count the remaining target cells, identified as ZsGreen positive cells, and then quantified as the proportion of live cells remaining over the total number of AML target cells (MOLM-13). All cytotoxicity experiments were performed in triplicates.

Flow cytometry analysis

Flow cytometric analysis was performed for the assessment of endogenous cell surface markers. Cells were stained with an antibody directly conjugated to a fluorescent dye while incubating in fluorescent-activated cell sorting (FACS) buffer for 30 minutes at room temperature. The cells were then washed and assessed for cell surface expression of specific antigens using an LSR

Fortessa X20 (BD Biosciences, San Jose, CA), followed by analysis using FlowJo v10 software (Tree Star). The transduction of FLT3 CAR in NK-92 cells was assessed by a flow cytometric assay measuring co-expressed GFP or anti-FLT3 single chain variable fragment (scFv) after staining transduced cells with an anti-mouse IgG F(ab) antibody. For the expression of the different forms of IL-15, the transduction efficiency was assessed through a co-expressed marker [i.e., truncated CD19 (tCD19)]. Finally, the transduction of FLT3 CAR in primary NK cells was evaluated through the co-expressed marker tCD19 or truncated EGFR (tEGFR). Information regarding the antibodies used for staining is included in **Supplementary Table 1.**

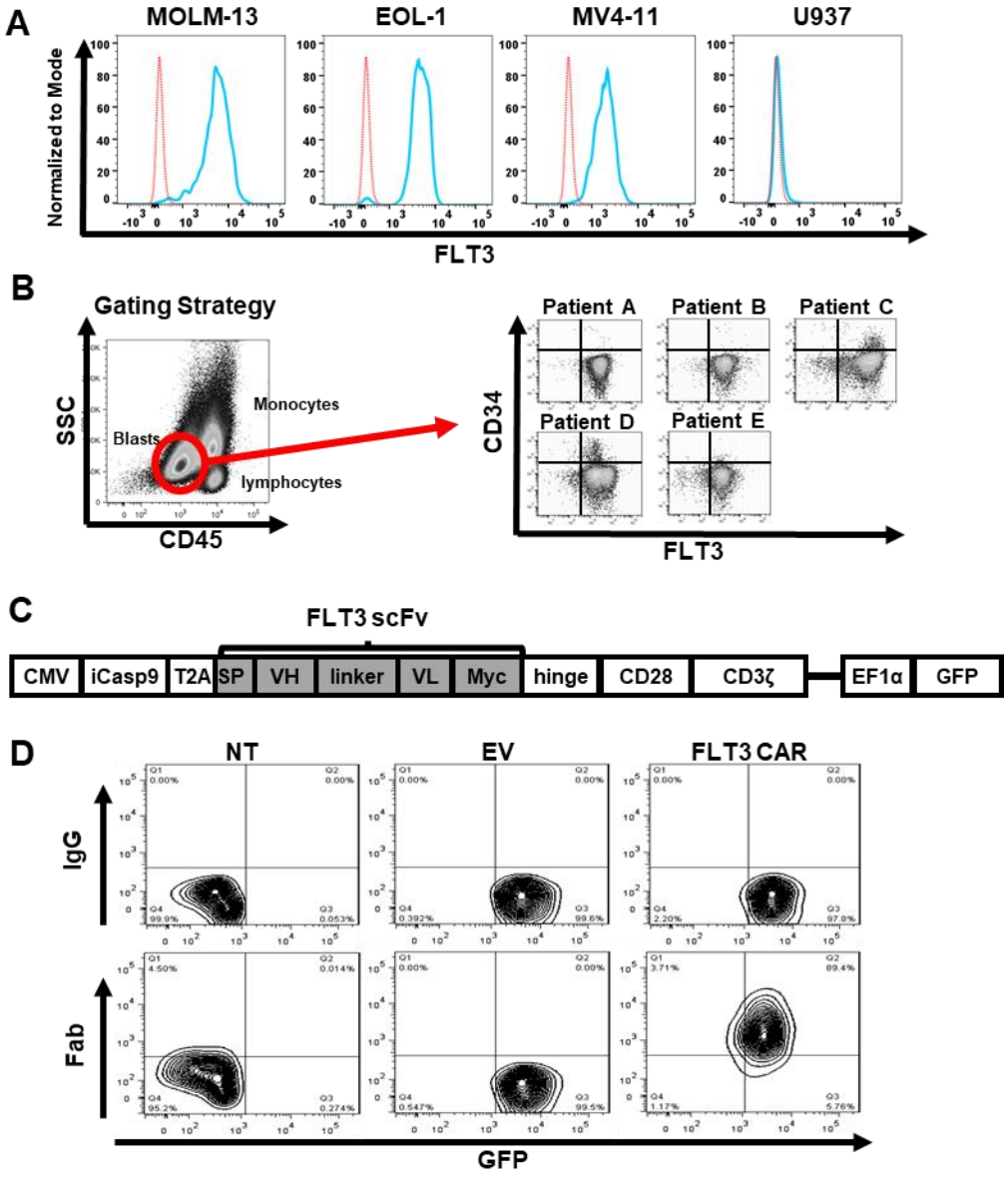
Study approval

Primary human AML patient samples were obtained from City of Hope Hematopoietic Tissue Biorepository. Peripheral blood samples of de-identified healthy donors were obtained from the Michael Amini Transfusion Medicine Center of City of Hope National Medical Center. Both types of samples were obtained under institutional review board–approved protocols. All animal experiments were approved by the City of Hope Institutional Animal Care and Use Committee.

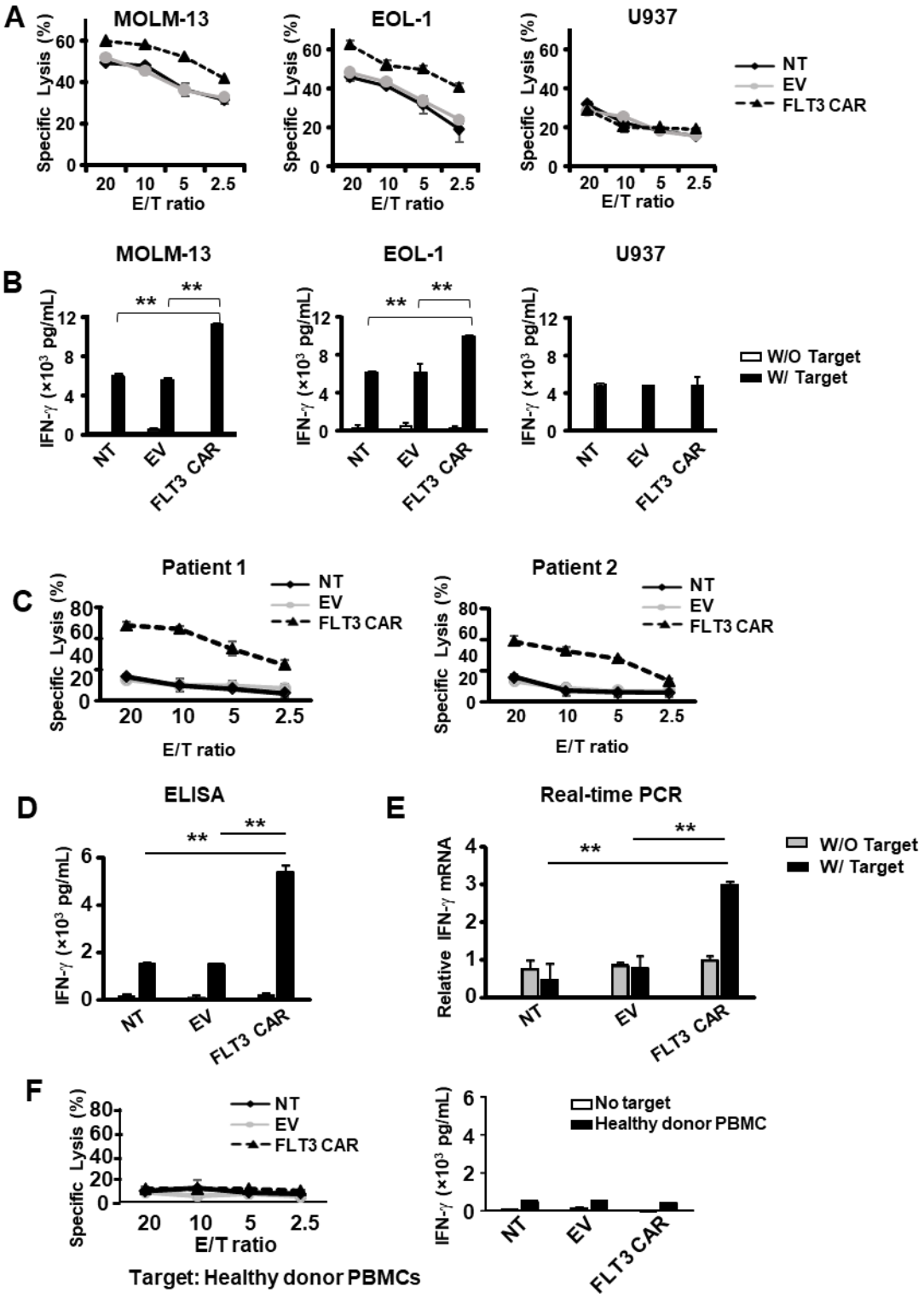
References

1. Chen L, Mao H, Zhang J, et al. Targeting FLT3 by chimeric antigen receptor T cells for the treatment of acute myeloid leukemia. *Leukemia*. 2017;31(8):1830-1834.
2. Lu T, Chen L, Mansour AG, et al. Cbl-B is upregulated and plays a negative role in activated human NK cells. *The Journal of Immunology*. 2021;206(4):677-685.

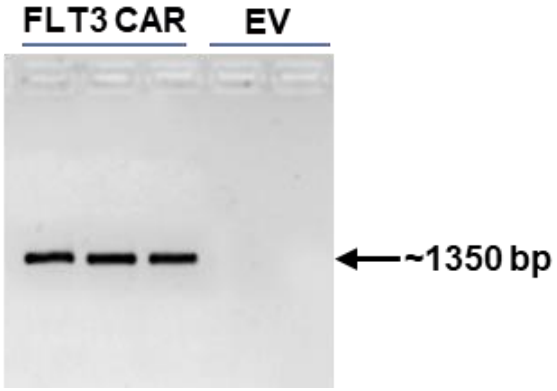
Supplementary figures and tables



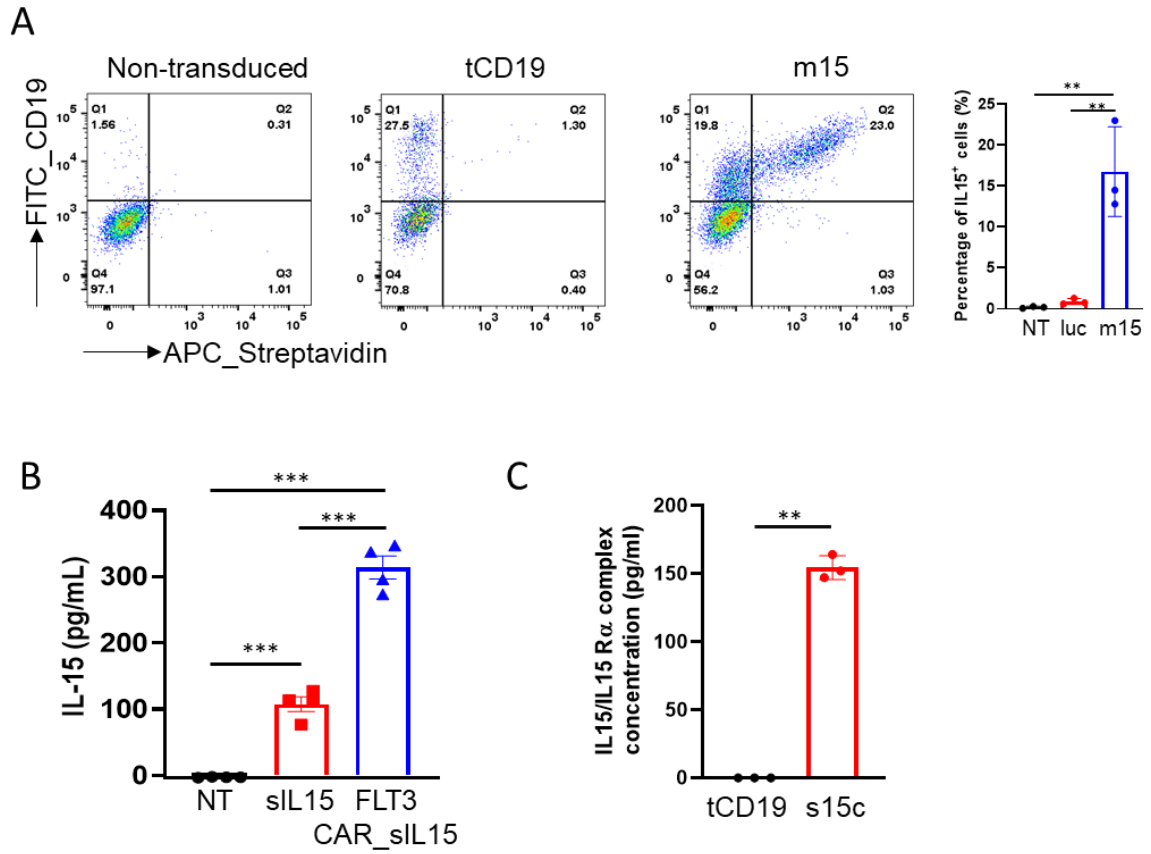
Supplementary Figure 1. Expression of FLT3 on AML cell lines as well as tumor blasts of AML patients and generation of an FLT3-CAR NK-92 cells. (A) The expression of FLT3 (blue) vs. isotype control (red) on the surface of the MOLM-13, EOL-1, MV4-11, and U937 AML cell lines. **(B)** High-density expression of FLT3 on AML blasts from 22 AML patients tested. Dashed lines, isotype control staining. Solid lines, FLT3 antibody staining. **(C)** Schematic representation of the generated FLT3-CAR lentiviral constructs. iCasp9, inducible caspase 9; T2A, a self-cleaving 2A gene; SP, signal peptide; VH, variable H chain; L, linker; VL, variable L chain. Myc, Myc tag; hinge, an IgG1 heavy chain hinge chain; CD28, a T cell co-stimulatory molecule; CD3ζ, CD3 zeta chain. **(D)** Parental non-transduced NK-92 (NT), NK-92 with an empty vector (EV), NK-92 cells transduced with FLT3-CAR (FLT3-CAR) were sorted based on Fab staining and GFP expression after cells were stained with biotin-labeled anti-mouse Fab-specific monoclonal antibody or an IgG control, followed by flow cytometry analysis.


A
MOLM-13
EOL-1
U937
Specific Lysis (%)
E/T ratio
NT
EV
FLT3 CAR
B
IFN-γ (×10³ pg/mL)
W/O Target
W/ Target
C
Patient 1
Patient 2
D
ELISA
E
Real-time PCR
Relative IFN-γ mRNA
F
No target
Healthy donor PBMC
Target: Healthy donor PBMCs

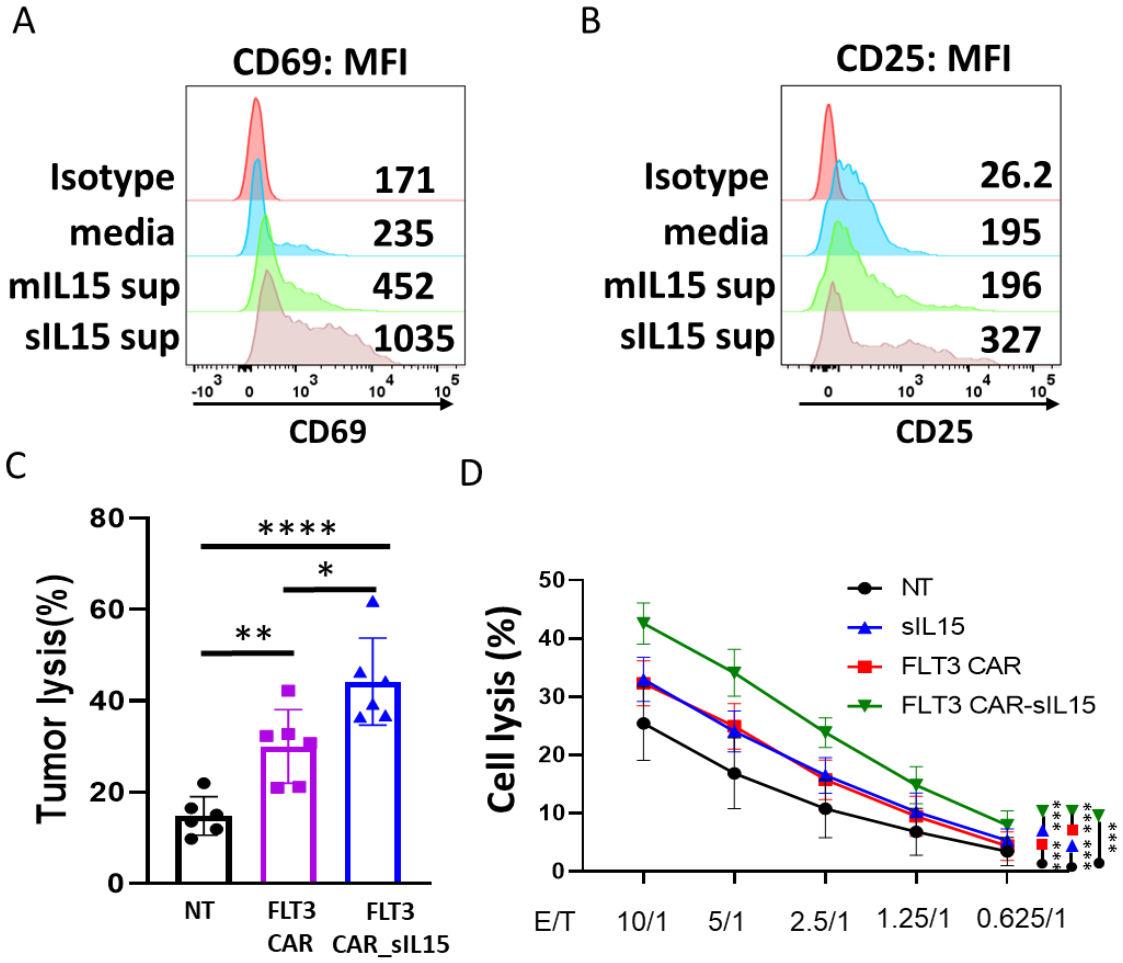
Supplementary Figure 2. Enhanced cytotoxicity and IFN-γ secretion of FLT3-CAR NK-92 upon recognizing FLT3$^+$ AML cells but not healthy PBMC. (A) Cytotoxic activity of non-transduced (NT) NK-92 cells, empty vector (EV) NK-92 cells, or FLT3-CAR NK-92 cells against MOLM-13, EOL-1 or U937 cells using a ^{51}Cr release assay. **(B)** IFN-γ secretion of NT NK-92 cells, EV NK-92 cells, or FLT3-CAR NK-92 cells in the presence or absence of MOLM-13, EOL-1, or U937 cells, analyzed by ELISA. **(C)** Cytotoxic activity of NT NK-92 cells, EV NK-92 cells, or FLT3-CAR NK-92 cells in the presence of patient PBMCs containing over 90% AML blasts, using a ^{51}Cr release assay. **(D** and **E)** IFN-γ secretion measured by ELISA (D) and IFN-γ mRNA assessed by real-time RT-PCR (E) in NT NK-92 cells, EV NK-92 cells, or FLT3-CAR NK-92 cells in the presence or absence of patient AML blasts. **(F)** Cytotoxicity and IFN-γ release of NT NK-92 cells, EV NK-92 cells, or FLT3-CAR NK-92 cells upon co-culture with PBMCs from healthy donors. Data shown represent the mean ± SEM. **, $p < 0.01$.



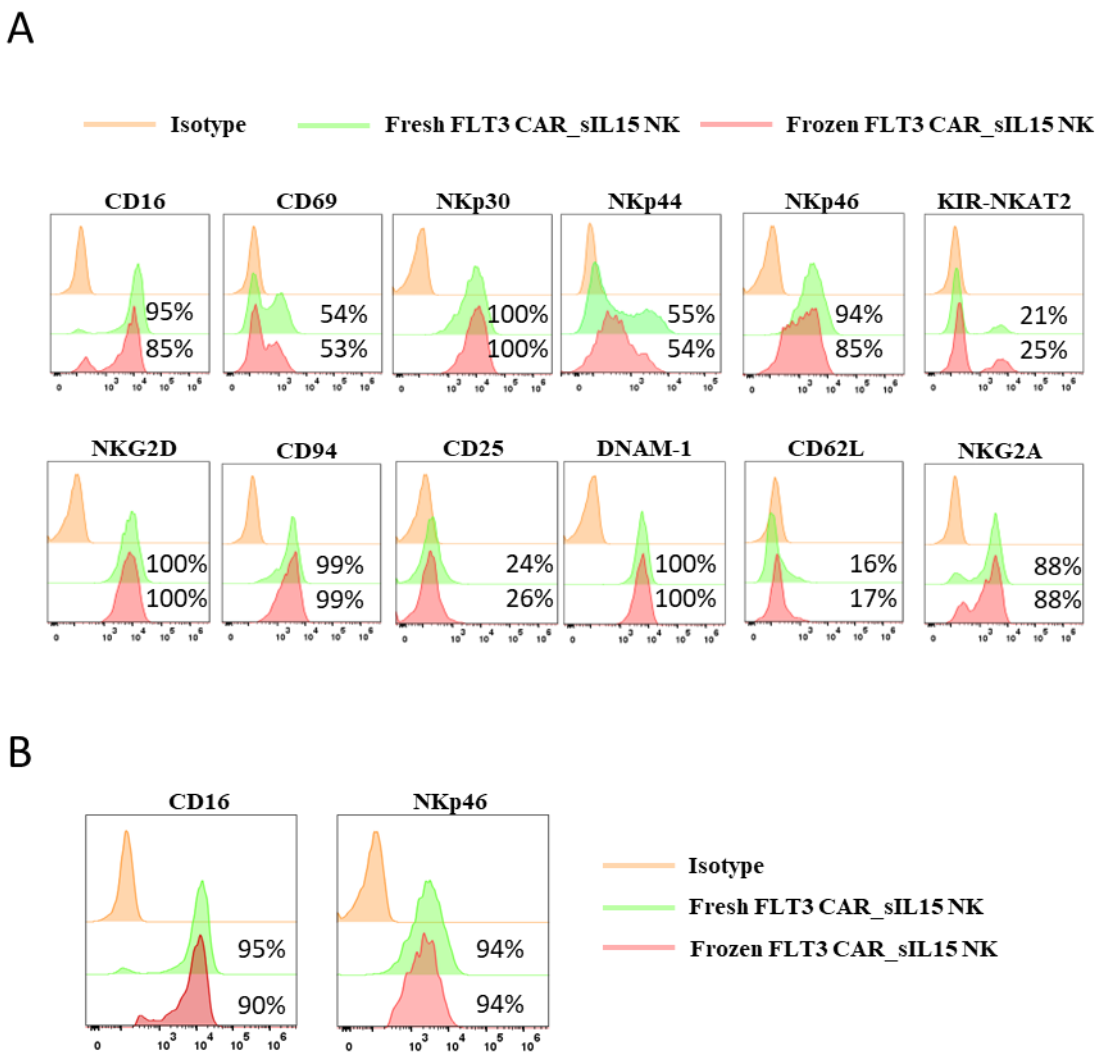
Supplementary Figure 3. The persistence of the FLT3 CAR NK-92 cells in vivo. PCR to show the persistence of the FLT3 CAR NK-92 cells at the time of sacrifice using primers specific for the FLT3 CAR.



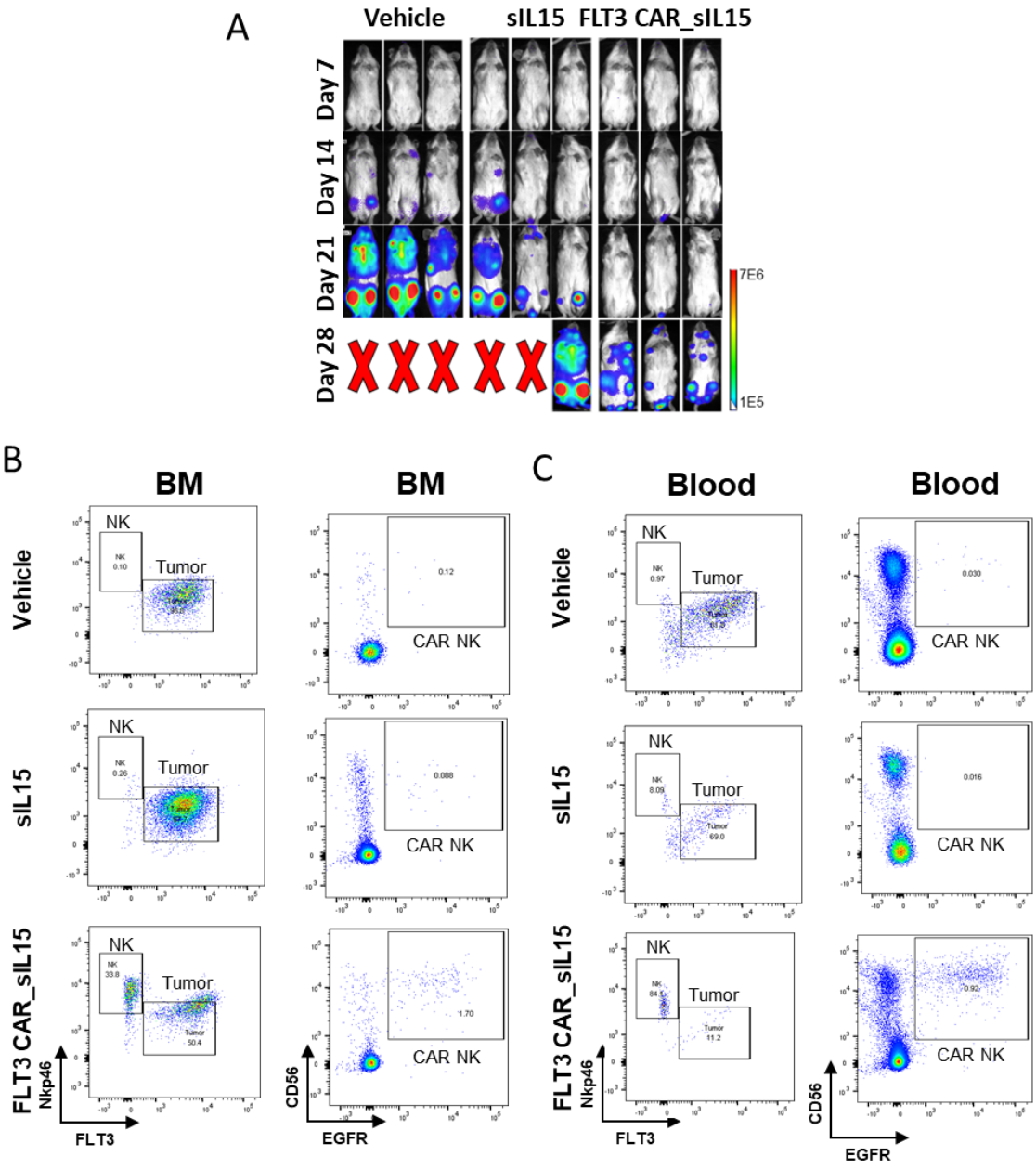
Supplementary Figure 4. The characteristics of different forms of IL-15 expressed in primary human cord blood NK cells. (A) IL15 and truncated (t)CD19 expression on non-transduced NK cells, tCD19-transduced NK cells, and mIL15 NK cells was detected by flow cytometry after the cells were stained with biotin-anti-IL15/APC-streptavidin and anti-CD19 antibodies. Representative flow cytometric plots of each group are shown in the left three panels and summary data are in the far right panel. Error bars indicate the standard deviations (SD), and statistical analyses were performed by one-way ANOVA with P values corrected for multiple comparisons by the Bonferroni method (n = 3 donors per group). **, $p < 0.01$. **(B)** IL-15 secretion by the engineered NK cells. IL-15 levels were quantified using ELISA using the supernatants of engineered NK cells (10^6) after 48-hr incubation (n = 4 donors). Data shown represent the mean ± SEM and statistical analyses were performed by one-way ANOVA with P values corrected for multiple comparisons by the Bonferroni method. **(C)** IL15/IL15 Rα secretion was detected by ELISA. Error bars indicate the standard deviations, and statistical analyses were performed by two-sided t test (n = 3 donors per group). *** , $p < 0.001$, **, $p < 0.01$.



Supplementary Figure 5. sIL-15 activates naïve NK and T cells. (A and **B)** Primary human cord blood NK cells expressing a soluble form of IL-15 (sIL15), or membrane-bound IL-15 (mIL15) were stimulated with K562 cells and their conditioned media (CM) was collected to culture naïve $CD56^+$ NK (A) or $CD3^+$ T cells (B) enriched from healthy individuals' peripheral blood. FACS analysis was performed on naïve NK or T cells cultured in fresh media or mIL15 or sIL15 CM, assessing the activation marker CD69 on NK cells (A) and the activation marker CD25 on T cells (B). **(C)** FLT3 CAR or FLT3 CAR_sIL15 NK cells were co-cultured with the AML cell line MOLM-13 for 48 hours and then subjected to flow cytometry–based cytotoxicity analysis at an E/T ratio of 4:1 (n = 5 donors). Data shown represent the mean ± SEM. **(D)** The cytotoxicity of non-transduced (NT), sIL15 NK, FLT3 CAR, and FLT3 CAR_sIL15 NK cells against MOLM-13 (n = 3 donors each), accessed by Cr^{51}-based cytotoxicity assay at indicated E/T ratios. A linear mixed model was used to compare matched groups and P values were corrected for multiple comparisons by Holm's method (C and D). Data shown represent the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

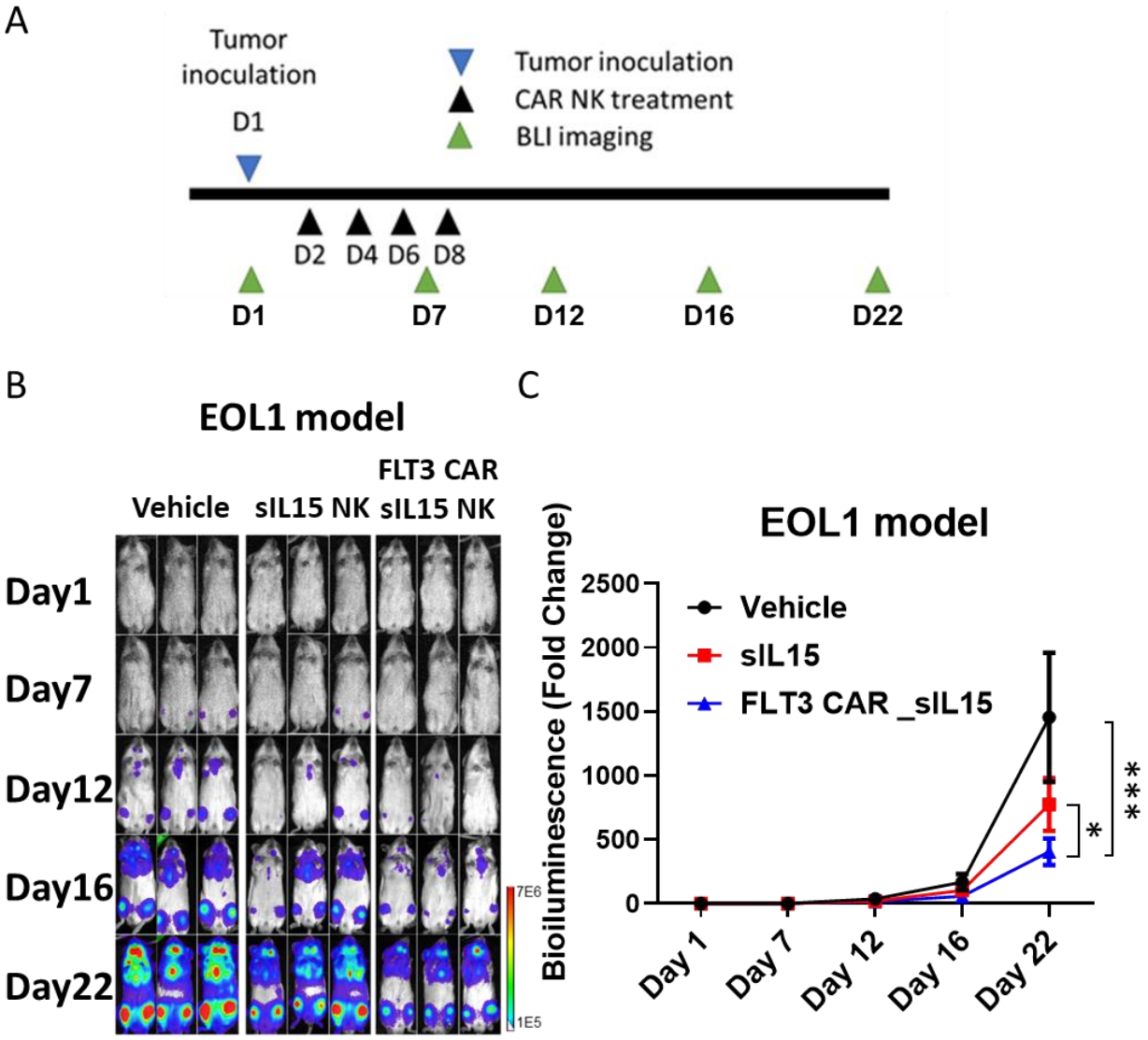


Supplementary Figure 6. Immunophenotype of fresh and frozen FLT3 CAR_sIL15 NK cells. **(A** and **B)** Native receptors on the FLT3 CAR_sIL15 NK cells were assessed by flow cytometry before and after one week of freezing. FACS analysis was performed right after cells were thawed (A) or after overnight culturing (B).

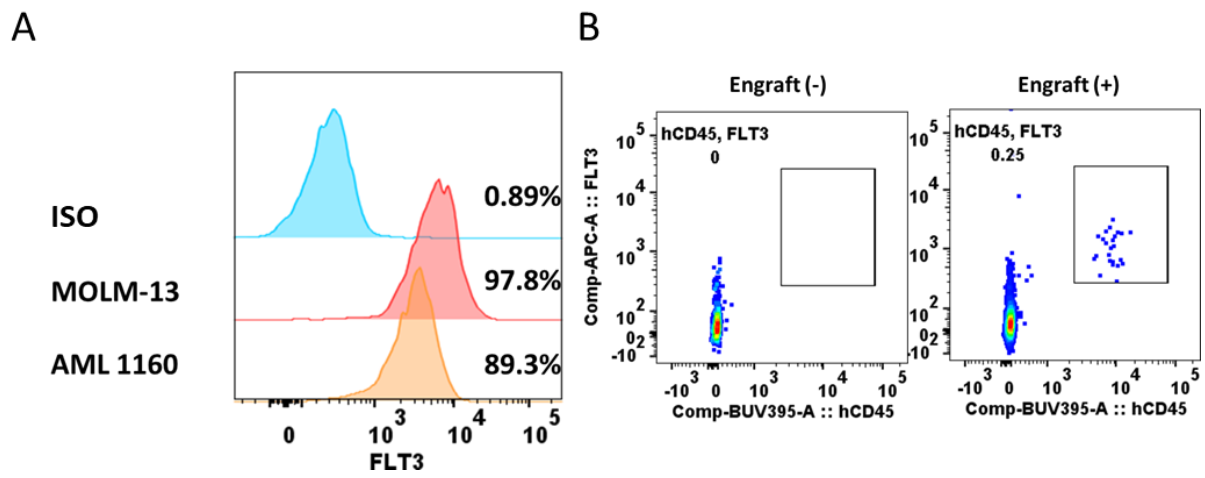


Supplementary Figure 7. Monitoring AML and NK cells from vehicle–, sIL15 NK–, or FLT3 CAR _sIL15 NK–treated mice. NSG mice engrafted with the FLT3^{+} MOLM-13 AML cells with "off-the-shelf" sIL15 NK cells or FLT3 CAR_sIL15 NK cells or vehicle. MOLM-13 AML cells expressing luciferase (1×10^4 MOLM-13_luc cells) were injected into 12-week-old NSG mice on day 1, followed by an infusion of a corresponding treatment (1×10^7 NK cells/dose) on day 5 and

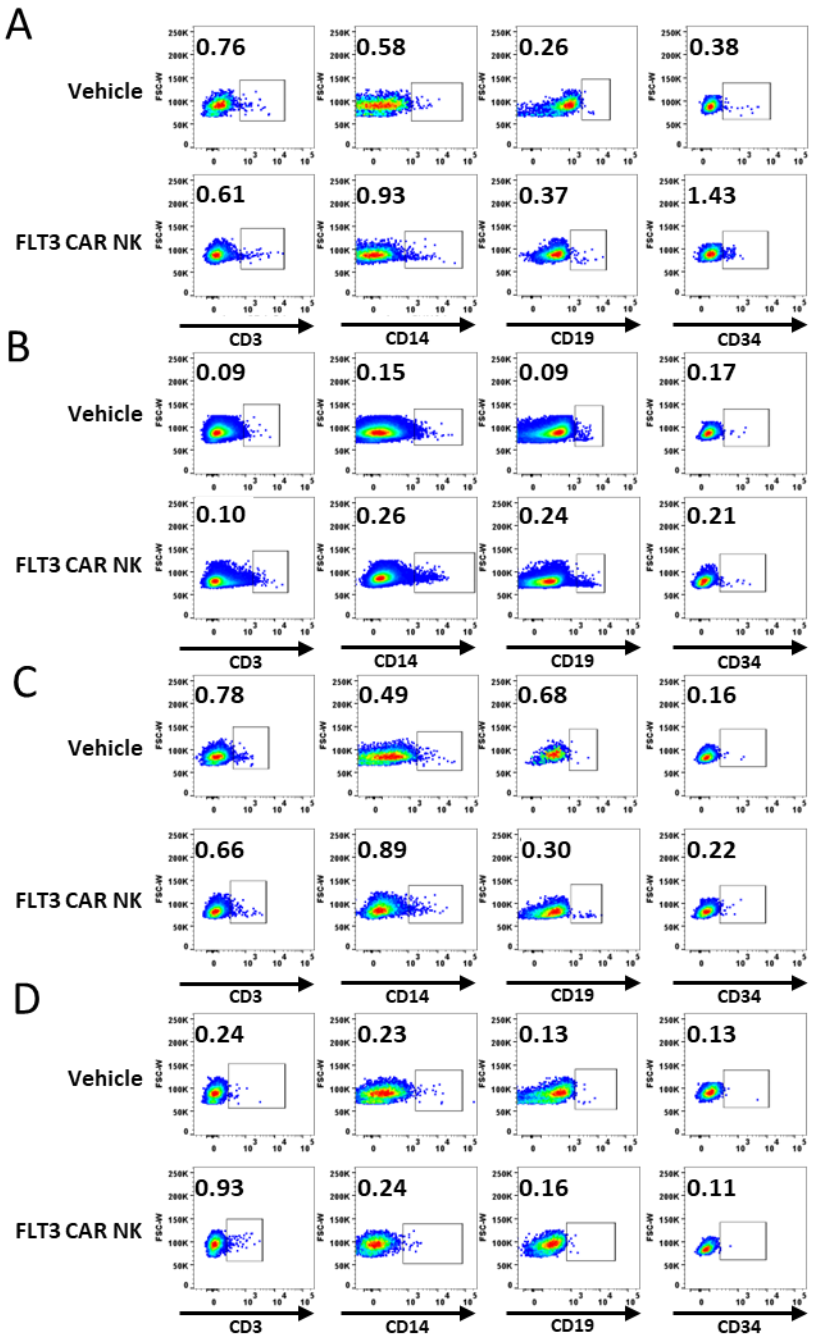
day 12; mice were sacrificed at day 21 and blood and bone marrow were assessed via flow cytometry for FLT-3$^+$ cells and NK cells. **(A)** Bioluminescence imaging was performed on days 7, 14, 21, and 28 to monitor tumor progression (n = 3/group). **(B** and **C)** The representative data of FACS analysis of the cells collected from bone marrow (B, left) or peripheral blood (C, right) of the mice treated with vehicle (freezing buffer), sIL15 NK cells, or FLT3 CAR_sIL15 NK cells. The population of tumor cells and that of NK cells were gated on human CD45 followed by CD135 (FLT3) cells and human CD45 followed by NKp46, respectively, while assessing expression of the CAR on NK cells was gated on CD56$^+$ and EGFR$^+$ (double positive for human CD56 and tEGFR). Data are representative of 3 mice per group.



Supplementary Figure 8. Off-the-shelf FLT3 CAR_sIL15 NK cells in the EOL-1 xenograft AML model. (A) Scheme of the EOL-1 AML model with indicated treatments. 5 × 10^5 EOL-1 cells expressing luciferase were injected into 8-10 weeks old male NSG mice on day 1. Vehicle or off-the-shelf NK cells (1 × 10^7) expressing the soluble form of IL-15 (sIL15) or FLT3-CAR_s15 were injected through tail veins on days 2, 4, 6, and 8. **(B)** Bioluminescence imaging was performed on the indicated days (n = 3 per group). **(C)** Quantification of ventral bioluminescence images is shown in (B). Data shown represent the mean ± SEM. ***, $p < 0.001$, *, $p < 0.05$.



Supplementary Figure 9. Treatment of a PDX model with FLT3 CAR_sIL15 NK cells. (A) FLT3 expression on the MOLM-13 AML cell line and primary AML blasts of a human donor (1160). **(B)** NSG mice were engrafted with 8×10^5 cells from a patient with FLT3^{+} AML. Twenty-one days later, the engraftment was confirmed by the detection of a human CD45^{+} FLT3^{+} AML cell population.



Supplementary Figure 10. FACS analysis of AML engrafted mice treated with FLT3 CAR_sIL15 NK cells. (A-D) CD34 inoculated NSGS mice were engrafted with FLT3$^+$ MOLM-13 AML cells and then treated with frozen and thawed FLT3 CAR_sIL15 NK cells. Mice were euthanized 21 days post tumor injections and human lymphocytes were isolated from the bone marrow (A), liver (B), spleen (C), and peripheral blood (D) for FACS analysis of CD19$^+$, CD3$^+$, CD34$^+$, and CD14$^+$ cells.

Supplementary Table 1. The antibodies used for flow cytometry

Antibodies	Source	Identifier
CD3	BD	Clone UCHT1
CD16	BD	Clone 3G8
TRAIL	BD	Clone RIK-2
CD62L	BD	Clone DREG-56
NKp46	BD	Clone 9E2
CD69	BD	Clone FN50
CD25	BD	Clone M0A251
CD94	BD	Clone HP-3D9
NKG2D	BD	Clone 1D11
KIR-NKAT2	BD	Clone DX27
DNAM-1	BD	Clone DX11
NKp44	Miltenyi	Clone 2.29
NKG2A	Miltenyi	Clone REA110
CD45	Biolegend	Clone 2D1
NKp30	Biolegend	Clone P30-15
EGFR	Biolegend	Clone AY13CD11c
FLT-3	BD	Clone 4G8
CD123	BD	Clone 7G3
CD19	BD	Clone HIB19
CD14	BD	Clone M5E2

References

1. Chen L, Mao H, Zhang J, et al. Targeting FLT3 by chimeric antigen receptor T cells for the treatment of acute myeloid leukemia. *Leukemia*. 2017;31(8):1830-1834.

2. Lu T, Chen L, Mansour AG, et al. Cbl-B is upregulated and plays a negative role in activated human NK cells. *The Journal of Immunology*. 2021;206(4):677-685.