Immune checkpoint molecule DNAM-1/CD112 axis is a novel target for natural killer-cell therapy in acute myeloid leukemia

Yuta Kaito,[1] Emi Sugimoto,[2] Fumi Nakamura,[3] Yutaka Tsukune,[4] Makoto Sasaki,[4] Shunsuke Yui,[5] Hiroki Yamaguchi,[5] Susumu Goyama,[6] Yasuhito Nannya,[1] Kinuko Mitani,[3] Hideto Tamura[7] and Yoichi Imai[3]

[1]Division of Hematopoietic Disease Control, Institute of Medical Science, The University of Tokyo, Tokyo; [2]Division of Cellular Therapy, Institute of Medical Science, The University of Tokyo, Tokyo; [3]Department of Hematology and Oncology, Dokkyo Medical University, Tochigi; [4]Department of Hematology, Juntendo University School of Medicine, Tokyo; [5]Department of Hematology, Nippon Medical School, Tokyo; [6]Division of Molecular Oncology, Graduate School of Frontier Sciences, The University of Tokyo, Tokyo and [7]Division of Diabetes, Endocrinology and Hematology, Department of Internal Medicine, Dokkyo Medical University Saitama Medical Center, Saitama, Japan.

Correspondence: Y. Imai
imaiyo-tky@umin.ac.jp

Received: February 8, 2023.
Accepted: September 8, 2023.
Early view: September 21, 2023.

https://doi.org/10.3324/haematol.2023.282915

Supplementary Data

Supplementary Methods

Cell lines and culture conditions

The leukemia cell lines (MV-4-11, THP-1, KG-1, K562, NB-4, Kasumi-1, and TF-1), and NK cell line (NK-92) were obtained from the American Type Culture Collection (VA, USA). The leukemia cell line MOLM-13 was obtained from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

MOLM-13, MV-4-11, THP-1, KG-1, K562, NB-4, Kasumi-1, and K562 cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemicals, Osaka, Japan) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, Merck KGaA, MD, USA) and 1% penicillin streptomycin (P/S; FUJIFILM Wako Pure Chemicals) at 37°C with 5% CO_2. TF-1 cells were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemicals) containing 10% FBS, 1% P/S, and 2 ng/mL recombinant human GM-CSF (rhGM-CSF; PeproTech, NJ, USA). NK-92 was cultured in α-MEM medium (FUJIFILM Wako Pure Chemicals) containing 12.5% FBS, 12.5% horse serum (Gibco, CA, USA), 1% P/S, 0.1 mM 2-mercaptoethanol (Gibco), 0.2 nM inositol (FUJIFILM Wako Pure Chemicals), 0.02 mM folic acid (FUJIFILM Wako Pure Chemicals), and 20 ng/mL recombinant human Interleukin-2 (rhIL-2; PeproTech, NJ, USA), NK-92 medium, at 37°C with 5% CO_2.

Flow Cytometry

PBS containing 0.5% bovine serum albumin (BSA; Nacalai Tesque, Kyoto, Japan) and 1 mM ethylenediaminetetraacetic acid (EDTA; Dojindo Wako Pure Chemicals, Kumamoto, Japan) was used as FACS buffer. The cells were collected, washed with FACS buffer, and stained for surface antigens. Staining was performed using the antibodies listed in Supplementary Table 1, at 4°C for 30 min in the dark. The cells were then washed twice with FACS buffer and analyzed using a flow cytometer. For

subsequent analysis of cells using a flow cytometer, a BD FACSCelesta Flow cytometer (BD Biosciences, NJ, USA) was used. FlowJo software (ver. 10.8.0, FlowJo LLC, OR, USA) was used to analyze the results. The flow cytometry analysis was repeated at least three times.

Lentiviral production and transduction

Lenti-X293T cells (Clontech; Takara Bio, Shiga, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; FUJIFILM Wako Pure Chemicals) containing 10% FBS and 1% P/S at 37°C with 5% CO_2.

The lentiviral plasmid (CSII-EF-MCS) was purchased from the National BioResource Project (Japan). It is a third-generation plasmid. The lentiviral plasmid (CSII-EF-fLuc-2A-EGFP) was produced as described previously.[1] The lentiviral plasmid (CSII-EF-DNAM-1) was prepared using pcDNA3.1-DNAM-1 purchased from GenScript, by cloning DNAM-1 complementary DNA using polymerase chain reaction (PCR) and inserting it into CSII-EF-MCS. The lentiviral plasmid (CSII-EF-DsRed) was produced by PCR cloning DsRed from the pDsRed-Express2-N1 Vector (Clontech; Takara Bio) and inserted into CSII-EF-MCS. A lentiviral plasmid (CSII-EF-DNAM-1-DsRed) was generated by inserting DNAM-1 cDNA into CSII-EF-DsRed.

All fragment insertions were performed using the In-Fusion HD Cloning Kit (Clontech; Takara Bio), following manufacturer's protocol. Each lentiviral plasmid (5 μg) was transfected into Lenti-X293T cells using transfer plasmids (pMDLg/p. RRE (3 μg), pRSV-rev (1 μg)), and packaging plasmid (pMD.G (1 μg)) for 2 days at 37°C with 5% CO_2. Lentiviral particles were obtained by centrifuging the samples at 400 *g* for 5 min at 4°C, and the supernatant was collected. The supernatant was centrifuged, concentrated at 6000 *g* for at least 16 h, and used for transfection of the target cells.

For transduction, target cells were exposed to centrifugally concentrated lentiviral particles at a multiplicity of infection of 5, after 48-72 h, the target cells were collected, washed with PBS, and

cultured. After another 48-72 h, the transduced cells were sorted using a Cell Sorter SH800S (Sony, Tokyo, Japan) and used in the subsequent experiments.

Gene editing using the Crispr-Cas9 system

Sequences targeting TIGIT (Exon1), CD155 (Exon2, 3), and CD112 (Exon2) were obtained from previous reports[2] and CCTop-CRISPR/Cas9 target online predictor (https://cctop.cos.uni-heidelberg.de:8043/index.html).[3] Single guide RNA (sgRNA) was synthesized according to the protocol of the sgRNA In Vitro Transcription Kit (Clontech; Takara Bio). The synthesized sgRNA and Cas9 protein (Clontech; Takara Bio) were introduced into the target cells via electroporation using In Vitro Electroporator CUY21Pro-Vitro (NEPA GENE, Chiba, Japan). Conditions were as follows: NK-92 (Poring Pulse: voltage 275 V, pulse width 2 ms, pulse interval 50 ms, 1 time; Driving Pulse: voltage 20 V, pulse width 50 ms, pulse interval 50 ms, 10 times), MOLM-13, MV-4-11 and, KG-1 (pooring pulse: voltage 150 V, pulse width 1 ms, pulse interval 50 ms, 1 time; driving pulse: voltage 20 V, pulse width 50 ms, pulse interval 50 ms, 10 times). After 2 weeks of culturing, the cells in which the expression of the target molecule was KO were sorted using Cell Sorter SH800S (Sony) and used in the subsequent experiments.

MTT assay

NK-92/modified NK-92 cells were seeded at 5×10^3 cells/100 μL per well in 96 well microplates, cultured at 37°C with 5% CO_2. After 24 or 48 h, 10 μL of Cell Counting Kit-8 reagent (Dojindo) was added and allowed to react for 2 h. The absorbance of the samples was measured at 450 nm using a Nivo spectrophotometer (Perkin Elmer, MA, USA). (Average absorbance of NK cell line - Average absorbance of medium only) was calculated as 100% to compare NK-92 and modified NK-92.

Cytotoxicity assay

Leukemia cell lines (MOLM-13, MV-4-11, THP-1, KG-1, and K562) were stained with CellStain-CFSE for 30 min. Then, 2.0×10^5 leukemia cells (T: target) were mixed with NK-92/modified NK-92 (E: effector) at E/T ratios of 1, 5, and 10 in 24 well plates and co-cultured for 4 h at 37°C with 5% CO_2. After 4 h, the cells were collected, washed with PBS, and stained with CellStain-PI solution for 15 min at room temperature in the dark. The cells were then washed twice with FACS buffer and analyzed using flow cytometry.

Degranulation assay

The leukemia cell lines were stained with CFSE for 30 min. The stained leukemia cell lines were mixed with 5.0×10^5 NK-92/modified NK-92 cells in 500 μL of NK-92 medium and seeded into 24 well plates at 37°C with 5% CO_2. One microliter of anti-APC-CD107a antibody (BioLegend, CA, USA) was added, and the cells were cultured for 1 h. After 1 h, monensin (BioLegend) was added and the cells were co-cultured for another 3 h. The cells were collected and washed twice with FACS buffer, and the CD107a positive percentage of CFSE-negative NK-92/modified NK-92 cells was analyzed using flow cytometry.

Intracellular staining (cytokine assay)

Leukemia cell lines were stained with a solution of CFSE 1 mM for 30 min. After staining, 5.0×10^5 leukemia cells and 5.0×10^5 NK-92/modified NK-92 cells were mixed in 500 μL NK-92 medium and seeded into 24 well plates at 37°C with 5% CO_2. One hour later, monensin was added, and cells were cocultured for another 3 h. After 3 h, the cells were collected and washed twice with PBS and dissolved in IC Fixation Buffer (eBioscience, MA, USA) in the dark at room temperature for 30 min. The cells were washed with 1× permeabilization buffer (eBioscience) and stained with anti-APC-TNFα

antibody (BioLegend) and anti-PE-Cy7-IFNγ antibody (BioLegend) for 30 min. The percentage of TNFα/IFNγ-positive CFSE-negative NK-92/modified NK-92 cells was analyzed using flow cytometry.

RNA sequencing processing

NK-92 and modified NK-92 cells were cultured to 1×10^7 cells/sample, and three samples were prepared by centrifugation at 400 *g* for 5 min at 4°C. The supernatant was collected by centrifugation and pelleted. The following experiments were outsourced to Macrogen(Tokyo, Japan).

RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), and the total RNA concentration was calculated using Quant-IT RiboGreen (Invitrogen, MA, USA). Libraries were independently prepared with 1 μg of total RNA from each sample using the Illumina TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc. CA, USA). cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen), and random primers, DNA Polymerase I, RNase H, and dUTP were used to synthesize cDNA. The cDNA was purified and enriched by PCR to produce a final cDNA library. Libraries were quantified using KAPA Library Quantification kits for Illumina Sequencing platform according to the qPCR Quantification Protocol Guide (KAPA BIOSYSTEMS, MA, USA), and qualitative assessment was performed using TapeStation D1000 ScreenTape (Agilent Technologies, CA, USA). Libraries were submitted to Illumina NovaSeq (Illumina, Inc.) and paired-end (2×100 bp) sequencing was performed by Macrogen. Read count values, and transcripts per million (TPM) values were obtained for each gene.

RNA sequencing analysis

Genes with a read count value of zero in at least one sample were excluded from further analysis as they were not expressed. Variable gene expression was analyzed using DESeq2, defined as fold-change (FC) ≥ 2 and, $P < 0.05$, and volcano plots were constructed. Rank files were created based on

P values, and the rank files were analyzed using GenePattern (https://www.genepattern.org/)[4]. Forty-seven NK cell-related genes were extracted based on previous reports.[5,6] Heat maps were generated using Morpheus (https://software.broadinstitute.org/morpheus/) by converting the TPM value of each gene into a Z-score. RNA sequencing data have been deposited in the DNA Data Bank of Japan (accession number DRA 015488).

Primary cells

Patients diagnosed with de novo AML at the University of Tokyo Institute of Medical Science Hospital, Nippon Medical School Hospital, Dokkyo Medical University Hospital, Dokkyo Medical University Saitama Medical Center, and Juntendo University Hospital were requested to participate in this study. Primary cells were collected from 7 mL peripheral blood or 5 mL bone marrow fluid. This study was reviewed and approved by the Ethics Review Committee of the Institute of Medical Science, University of Tokyo (2020-16-0623).

Analysis of primary cells

Peripheral blood or bone marrow fluid was hemolyzed using hemolysis buffer containing 0.15 M NH_4Cl (FUJIFILM Wako Pure Chemicals), 10 mM $KHCO_3$ (FUJIFILM Wako Pure Chemicals), and 0.13 mM EDTA. After lysis, the cells were washed with FACS buffer and stained for surface antigens. Staining was performed at 4°C for 30 min in the dark, and the cells were washed twice with FACS buffer. The cells were analyzed and sorted using BD FACS Aria II SORP (BD Biosciences).

Mouse

NSG mice were purchased from Charles River (Kanagawa, Japan). Mice aged 6–8-weeks were used in the experiments. All mice used in this study were bred and managed at the Laboratory Animal

Research Facility, Institute of Medical Science, University of Tokyo. Experiments were conducted in accordance with the Tokyo University Animal Experiment Practice Regulations and Tokyo University Animal Experiment Practice Manual. All experiments were approved by the Animal Experiment Committee of the Institute of Medical Science at the University of Tokyo (A20-25).

In vivo experiments with the xenograft model

A lentiviral plasmid (CSII-EF-fLuc-2A-EGFP) was used to introduce luciferase into the MOLM-13 cells. D-Luciferin was dissolved in PBS at 1.5 mg/mL. Ten minutes after intraperitoneal administration of d-luciferin, luminescence was assessed using IVIS. The mice were checked daily for signs of leukemia progression, including weight loss, decreased activity, and paralysis. Mice that developed symptoms were euthanized via carbon dioxide inhalation.

Statistical Analysis

Multivariate analysis with Cox proportional hazard model for TCGA data was performed using R (ver 4.3.1). All other statistical analyses were performed using GraphPad Prism software version 9.4.1 (GraphPad Software, MA, USA). Experimental data are presented as mean ± standard error (SE). The bars for the clinical sample data represent median. A two-tailed Student's t-test and one-way ANOVA was used to analyze statistically significant differences between groups 2 and 3. Tukey's test was used for multiple group comparisons. For clinical samples, Mann–Whitney U test and Wilcoxon signed-rank test were used to analyze statistically significant differences between the groups. Linear regression analysis was used for the correlation analysis. The log-rank test was used to analyze the survival curves. Multiple group comparisons were performed using Holm test. A P value of less than 0.05 indicated a significant difference.

Supplementary references

1. Futami M, Suzuki K, Kato S, et al. The novel multi-cytokine inhibitor TO-207 specifically inhibits pro-inflammatory cytokine secretion in monocytes without affecting the killing ability of CAR T cells. *PLoS One*. 2020;15(4):e0231896.

2. Stamm H, Klingler F, Grossjohann EM, et al. Immune checkpoints PVR and PVRL2 are prognostic markers in AML and their blockade represents a new therapeutic option. *Oncogene*. 2018;37(39):5269-5280.

3. Stemmer M, Thumberger T, Del Sol Keyer M, Wittbrodt J, Mateo JL. CCTop: An Intuitive, Flexible and Reliable CRISPR/Cas9 Target Prediction Tool. *PLoS One*. 2015;10(4):e0124633.

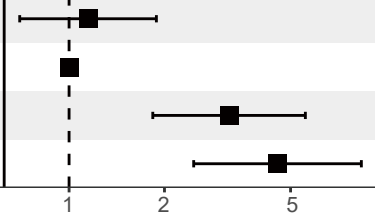
4. Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. *Nat Genet*. 2006;38(5):500-501.

5. Martinet L, Ferrari De Andrade L, Guillerey C, et al. DNAM-1 expression marks an alternative program of NK cell maturation. *Cell Rep*. 2015;11(1):85-97.

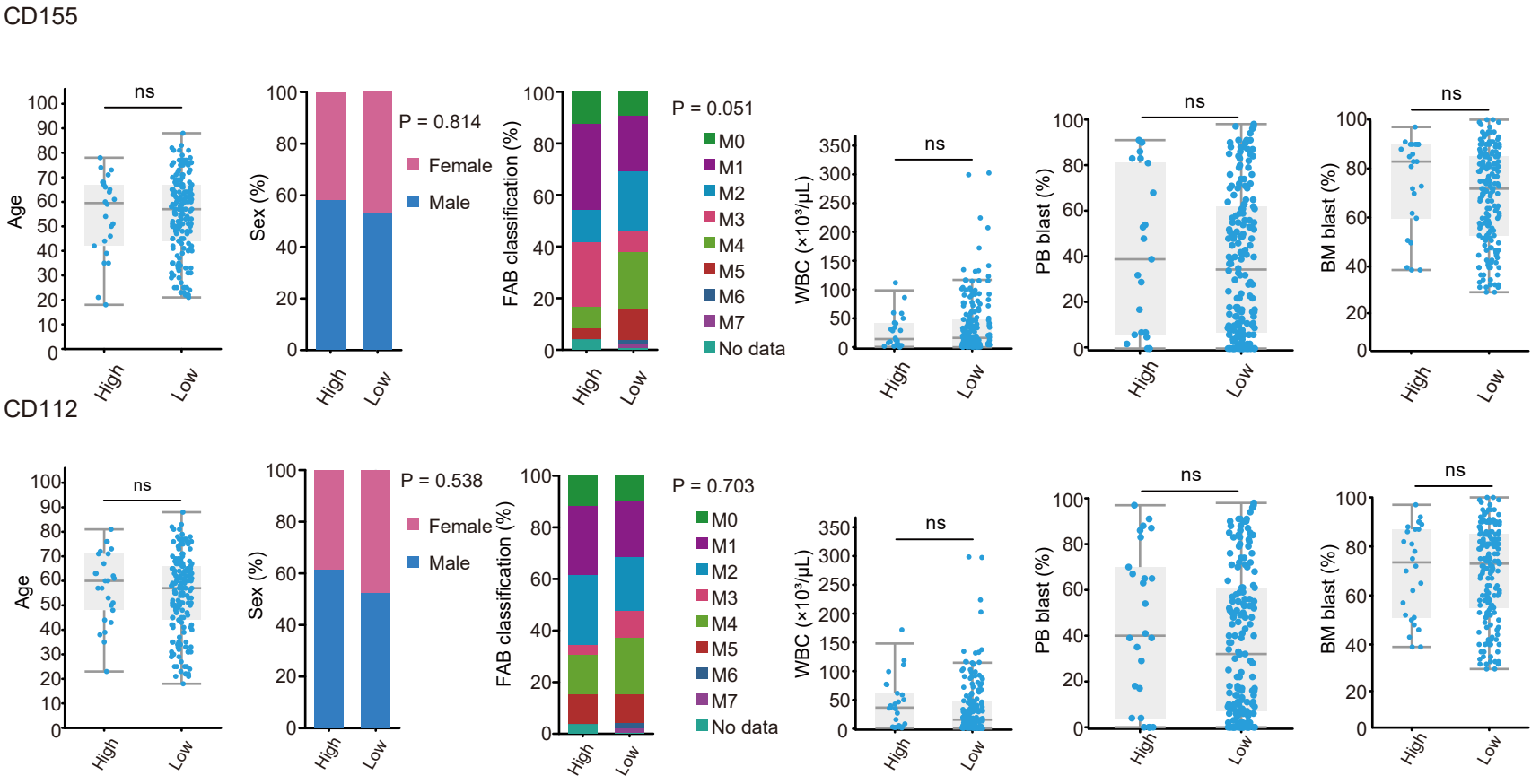
6. Landolina N, Mariotti FR, Ingegnere T, et al. IL-1R8 silencing improves the anti-tumor function of freshly isolated human NK cells. *J Immunother Cancer*. 2022;10(3).

A

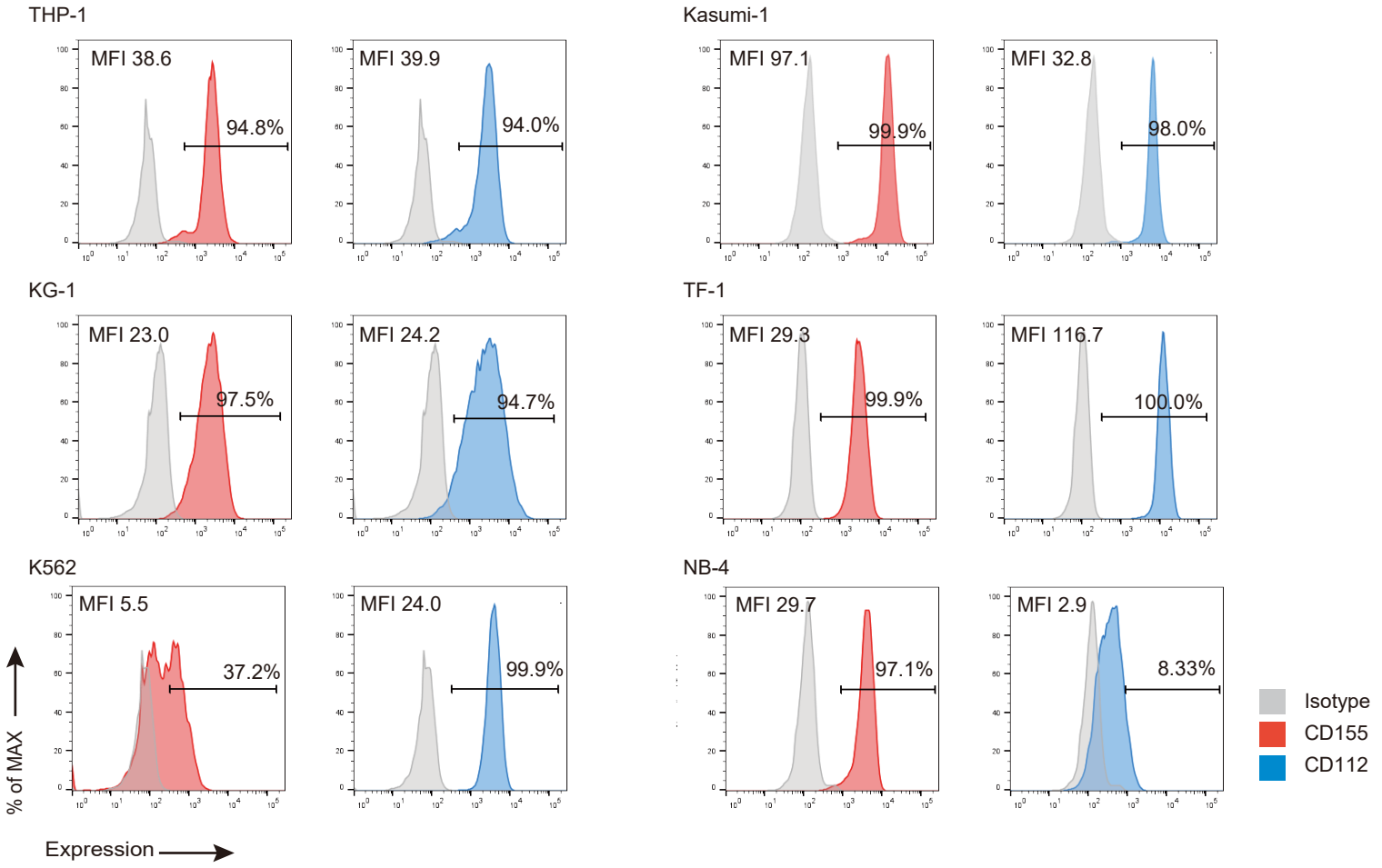
Variable		N	Hazard ratio	HR (95 % CI)	p
CD112	High	25		1.15 (0.70, 1.89)	0.6
Mol_risk	Good	39		Reference	
	Intermediate	106		3.20 (1.84, 5.57)	<0.001
	Poor	51		4.55 (2.47, 8.36)	<0.001



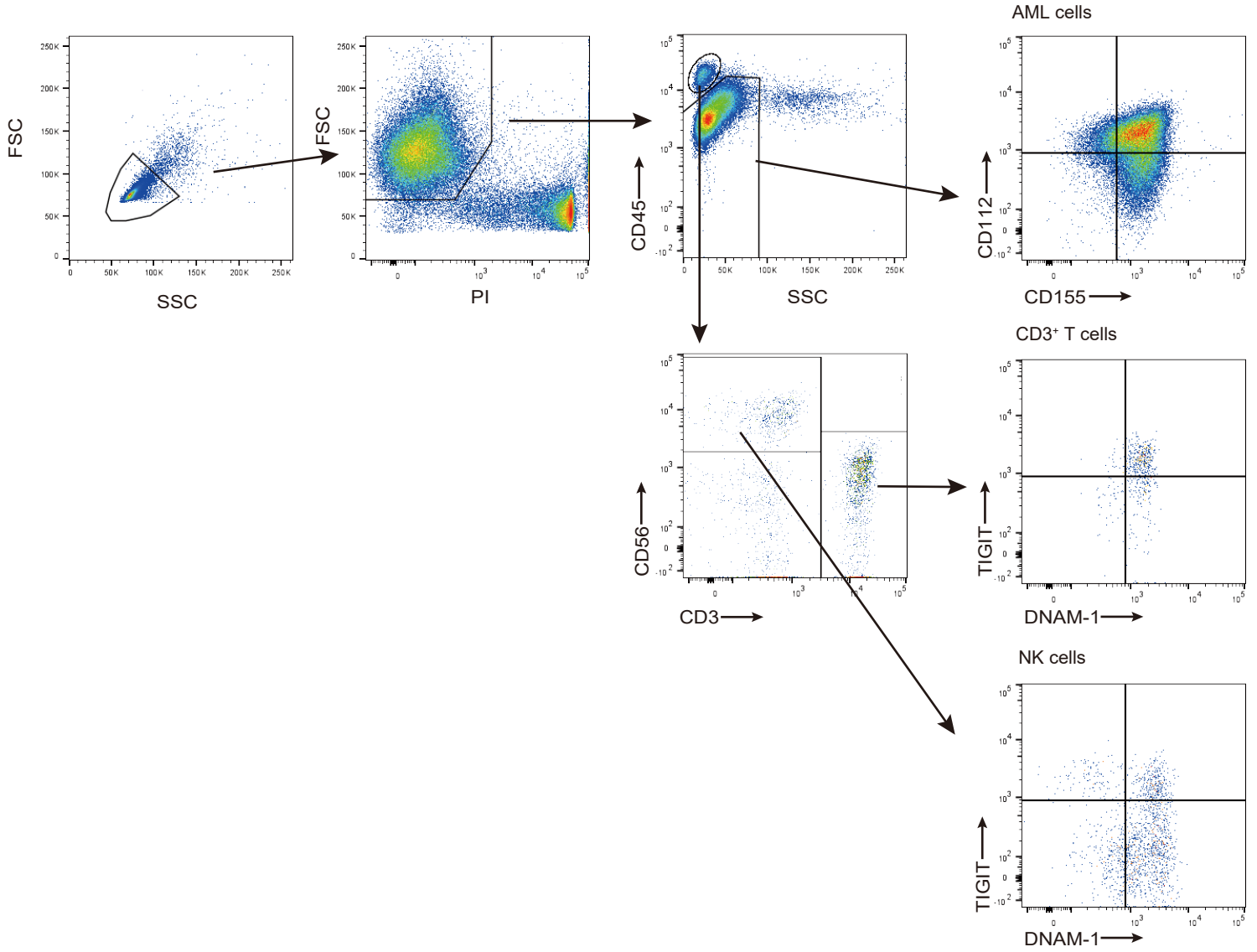
B



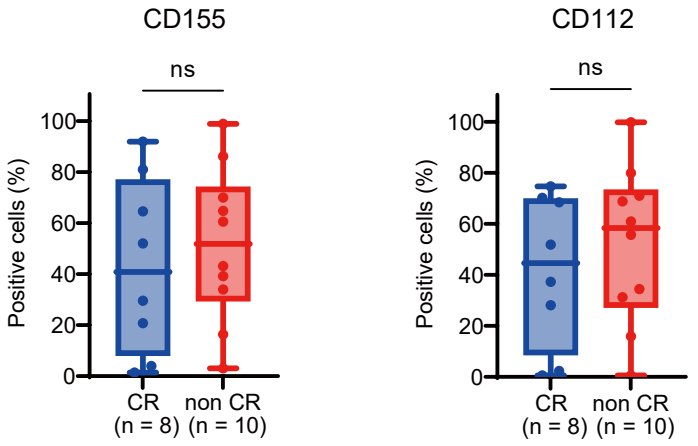
Supplementary Figure 1. Relationship between CD155 and CD112 expression and clinical features in patients with acute myeloid leukemia. (A) A Forest plot showing the result of a multivariate analysis for overall survival. 95% CI: 95% confidence interval, HR: hazard ratio. (B) The clinical features were compared in groups of patients with high or low CD155/CD112 expressions. All analyses were performed in cBioPortal. The chi-squared test was used for analyzing sex and FAB classification. Wilcoxon test was used for analyzing age, and WBC, PB blast (%), and BM blast (%). ns: no significant difference, FAB: French-American-British, WBC: white blood cell, PB: peripheral blood, BM: bone marrow.



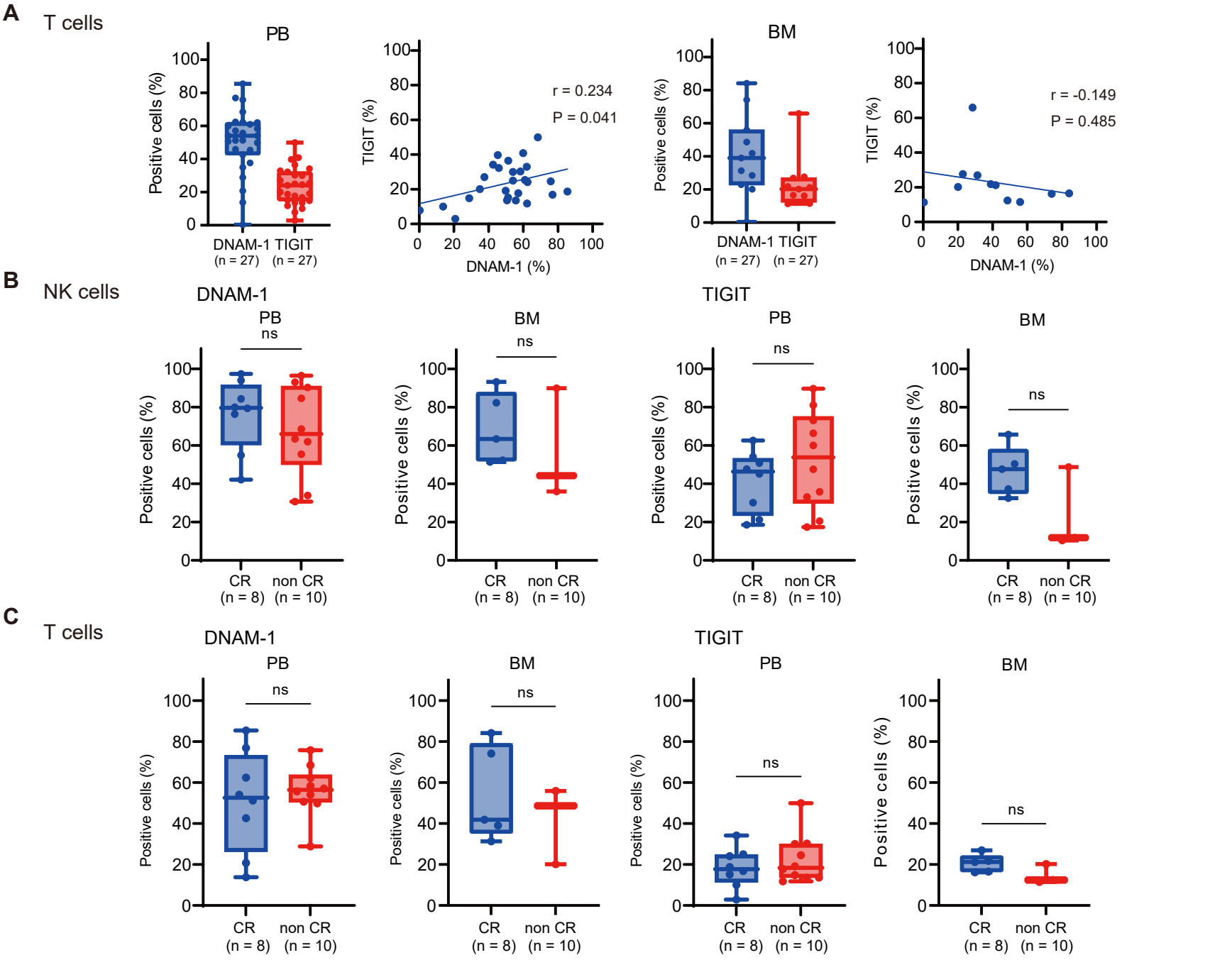
Supplementary Figure 2. CD155 and CD112 expression of leukemia cell lines. CD155 and CD112 expression in AML cell lines (THP-1, KG-1, NB-4, Kasumi-1, and TF-1) and in the blastic phase of chronic myeloid leukemia cell line (K562) were analyzed using flow cytometry. Gray, red, and blue histograms indicate isotype control, CD155, and CD112 expression, respectively.



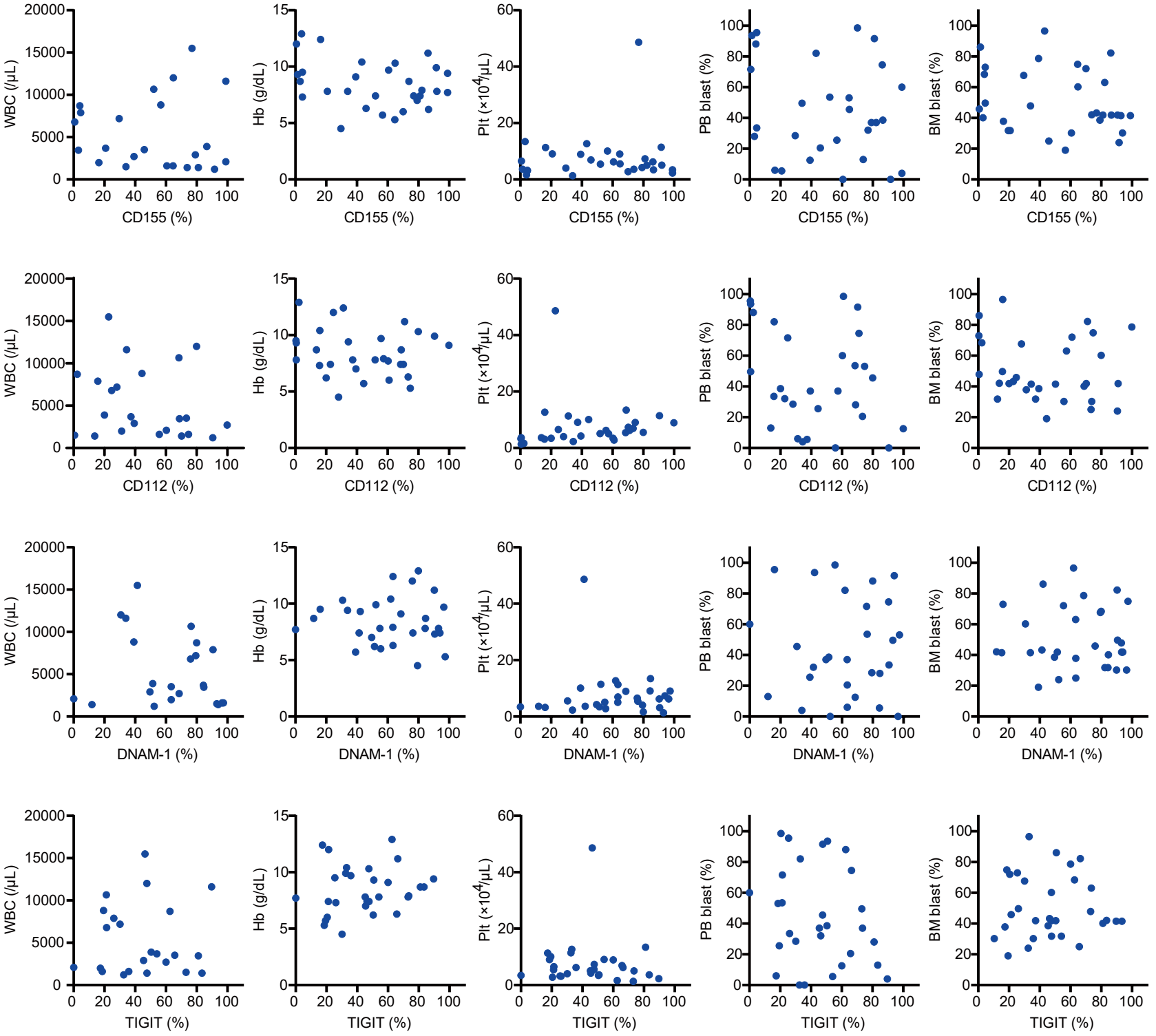
Supplementary Figure 3. Analysis of primary AML samples. Gating strategy for the analysis of primary AML samples. FSC, forward scatter; SSC, side scatter; PI: propidium iodide.



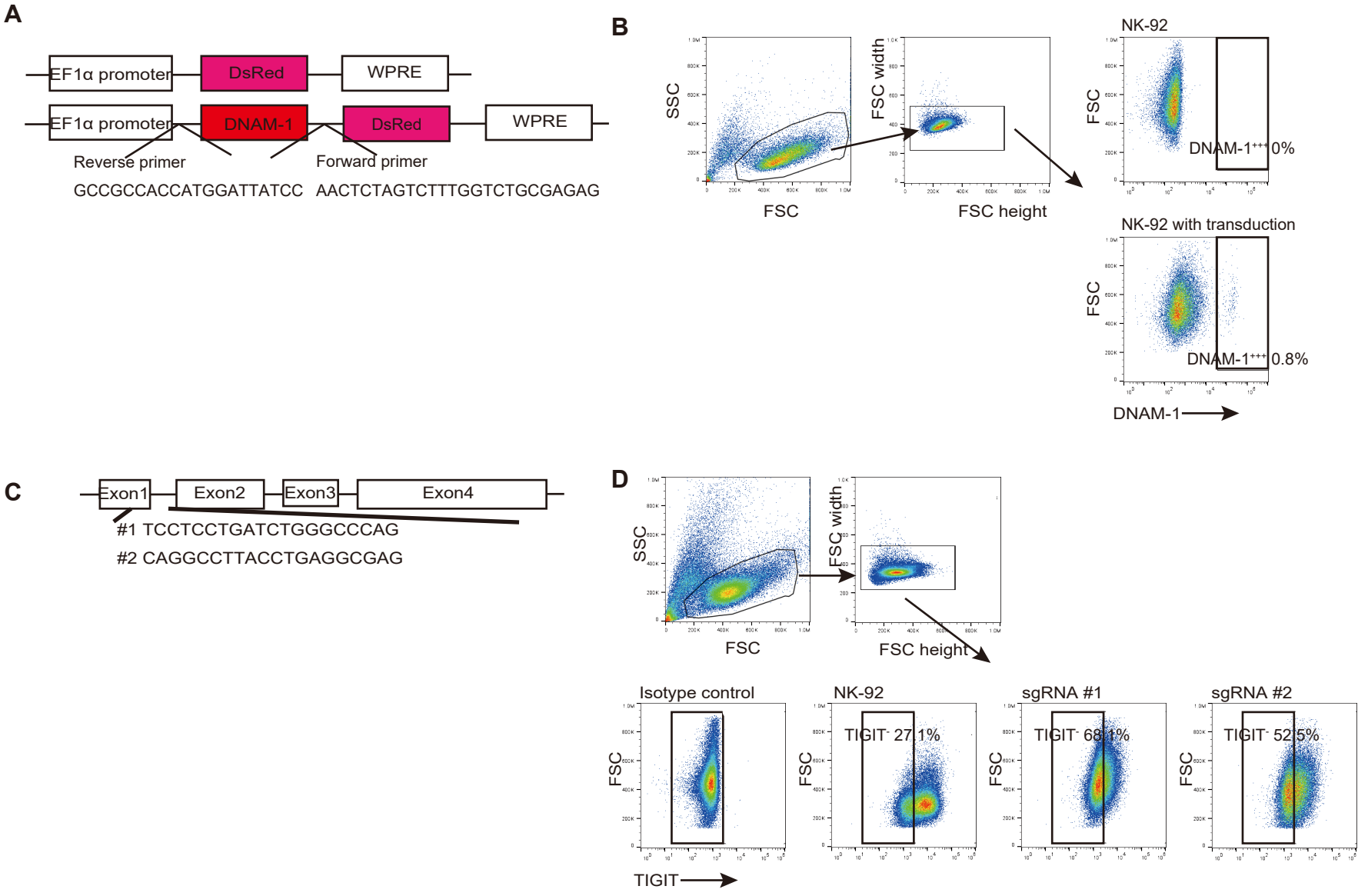
Supplementary Figure 4. Relationship between the expression of CD155/CD112 in AML blasts and their response to therapy. The percentage of CD155/CD112 positive AML cells in two groups of patients who achieved CR and non-CR after the initial treatment was analyzed using flow cytometry, and it is shown in box and whisker plots. Comparison between the groups was made using Mann-Whitney U test. ns: no significant difference.



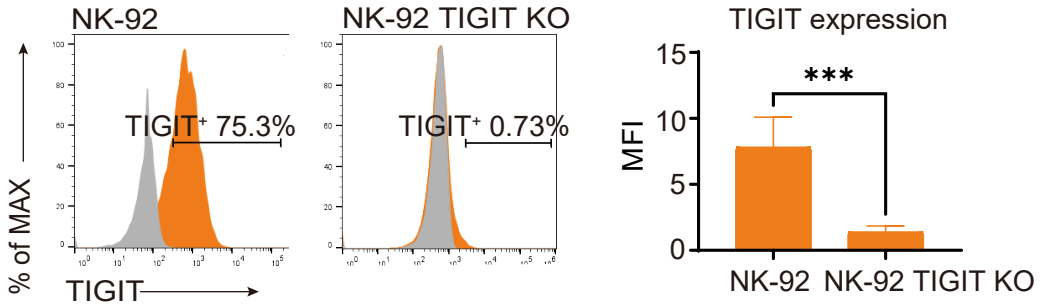
Supplementary Figure 5. Analysis of Nectin/Nectin-like family molecule receptors on NK and T cells. (A) Left: Flow cytometry analysis of the percentage of DNAM-1/TIGIT-positive T cells in peripheral blood and bone marrow fluid samples from patients with AML is shown in box and whisker plots. Right: scatter plots of percentage of DNAM-1 and TIGIT positive cells. The bars indicate regression lines. Linear regression analysis was used to analyze the correlations. (B) The percentage of DNAM-1/TIGIT positive NK cells in two groups of patients who achieved CR and non-CR after the initial treatment was analyzed using flow cytometry, and it is shown in box and whisker plots. Comparison between the groups was made using Mann-Whitney U test. (C) The percentage of DNAM-1/TIGIT positive T cells in two groups of patients who achieved CR and non-CR after the initial treatment was analyzed using flow cytometry, and it is shown in box and whisker plots. Comparisons between two groups were performed using Mann-Whitney U test. ns: no significant difference. r: correlation coefficient, PB: peripheral blood, BM: bone marrow.



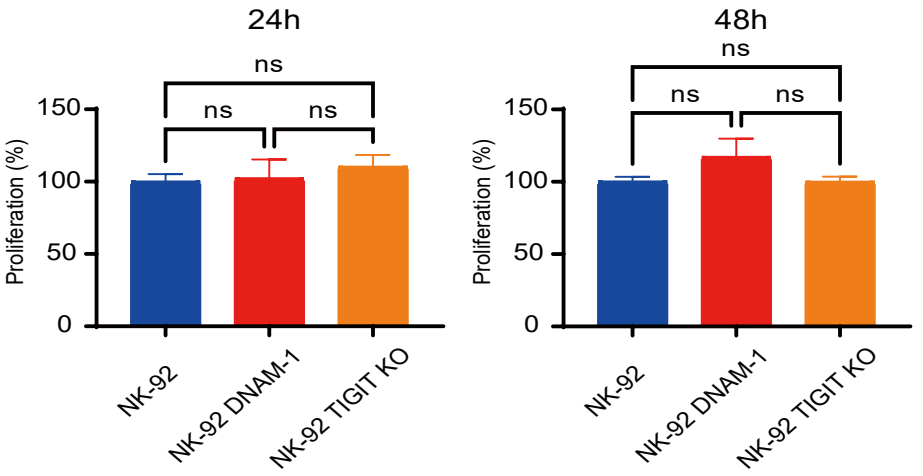
Supplementary Figure 6. Association of expression of Nectin/Nectin-like family molecules or their receptors with clinical test data. The associations between CD155, CD112, DNAM-1, and TIGIT respectively, and WBC count, Hb level, Plt level, PB blast (%), and BM blast (%) are shown. WBC: white blood cell, Hb: hemoglobin, Plt: platelet, PB: peripheral blood, BM: bone marrow.



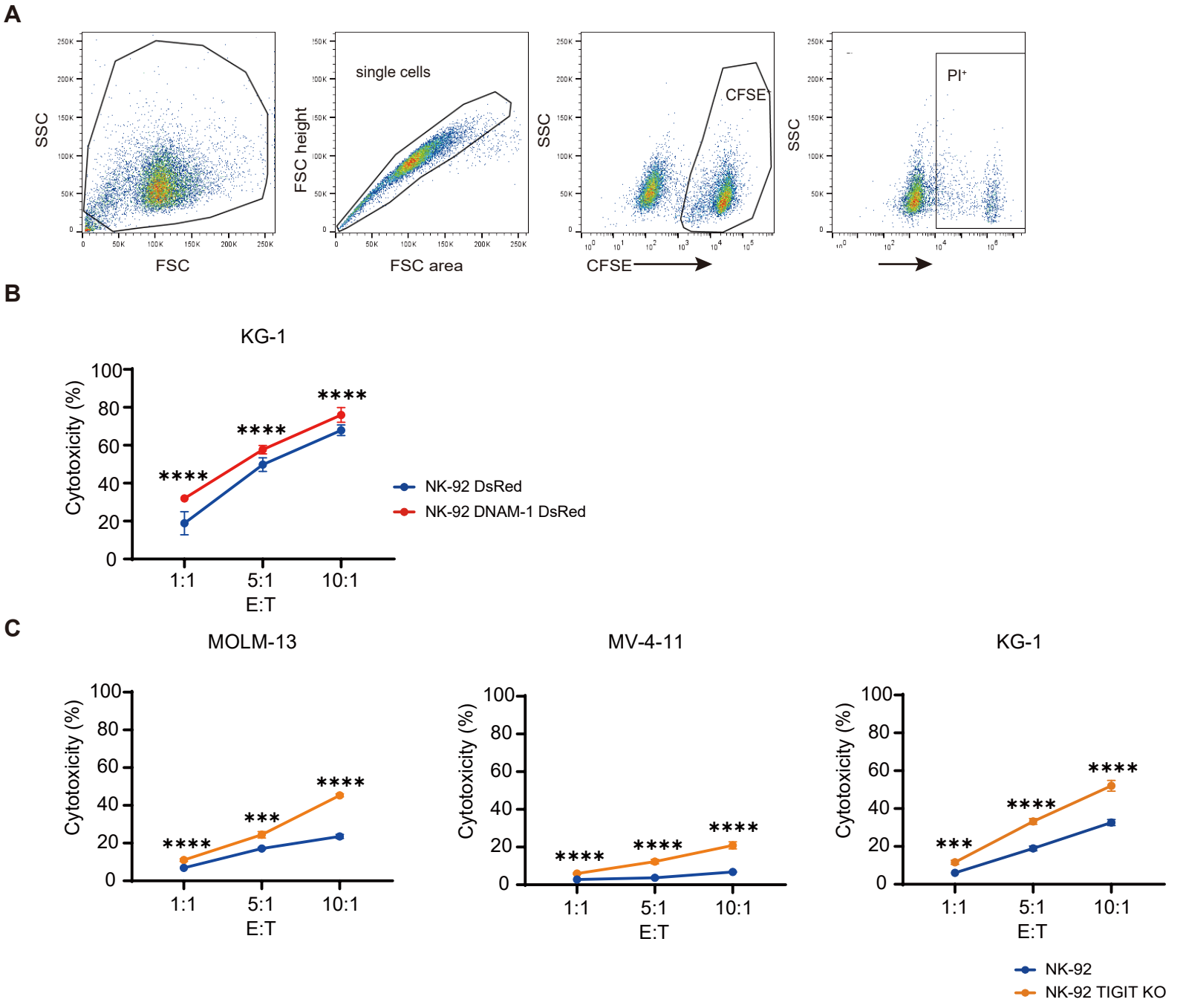
Supplementary Figure 7. Generation of NK-92 with modified receptors for Nectin/Nectin-like family molecules. (A) Schematic diagram of the lentiviral vector transfected with DNAM-1 cDNA and primer sequences used for synthesis using the In-Fusion HD Cloning kit. (B) Flow cytometry analysis after transduction with the lentivirus. DNAM-1 positive cells were sorted and expanded. (C) Target sequences of sgRNA used for TIGIT KO and its sites. (D) Flow cytometry analysis after TIGIT KO using the Crispr-Cas9 system. TIGIT-negative cells were sorted and expanded. FSC: forward scatter, SSC: side scatter.



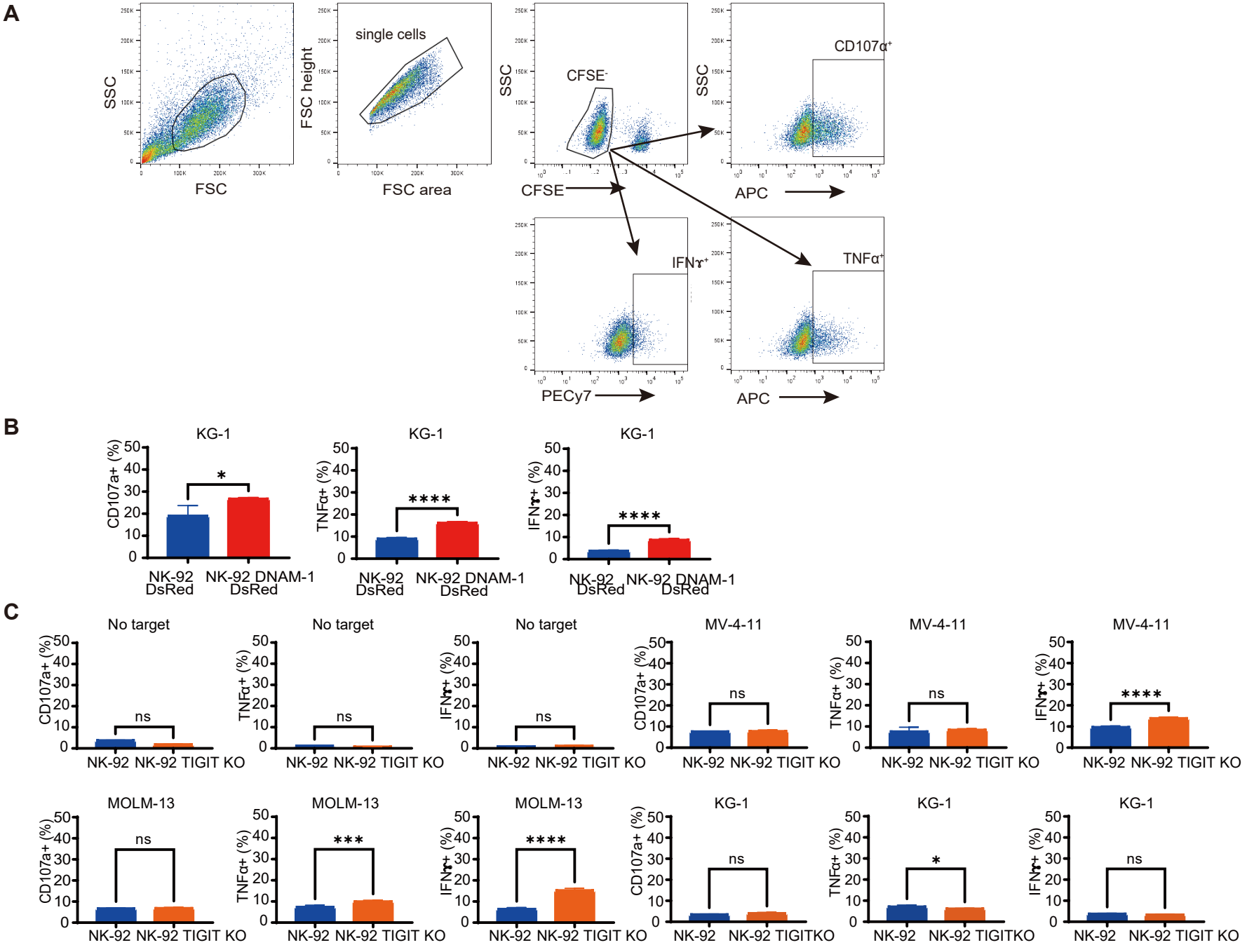
Supplementary Figure 8. Generation and functional analysis of NK-92 with modified receptors for Nectin/Nectin-like family molecules. TIGIT expression in of NK-92 and NK-92 TIGIT KO. Gray histograms indicate isotype control, orange indicates TIGIT expression. Comparison between the two groups was performed by using two-tailed Student' s t-test. Mean ± SE is shown. ns: no significant difference. ***P < 0.001, MFI: median fluorescence intensity



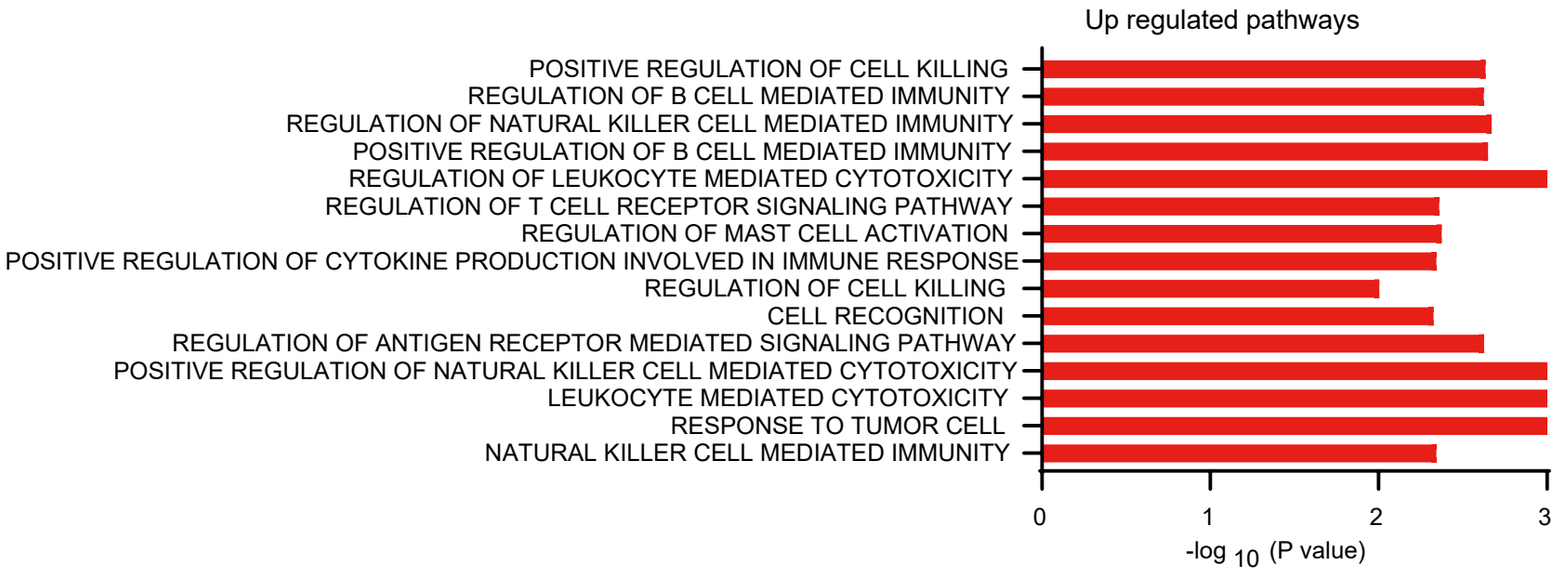
Supplementary Figure 9. Proliferation of NK-92 and modified NK-92. The proliferation of NK-92, NK-92 DNAM-1, and NK-92 TIGIT KO cells was compared using the Cell counting kit-8 assay. A one-way ANOVA variance was used to compare the three groups. Mean ± SE is shown. ns: no significant difference.

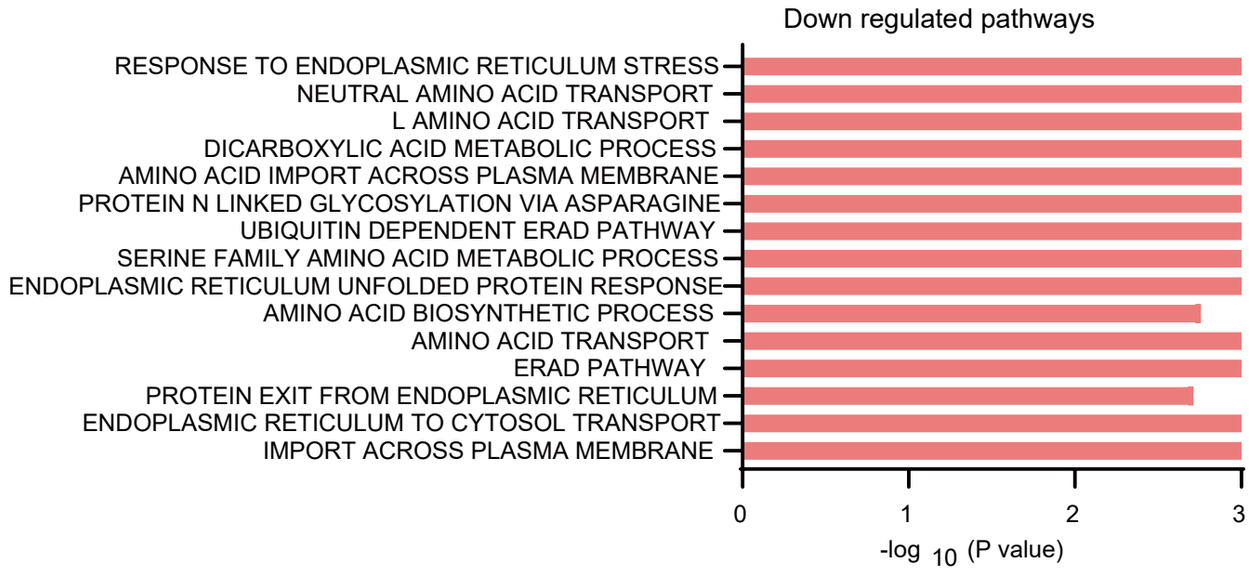


Supplementary Figure 10. Gating strategy for cytotoxicity assay. (A) Schematic diagram of cytotoxicity assay using flow cytometry. FSC, forward scatter; SSC, side scatter; CFSE, carboxyfluorescein succinimidyl ester; PI, propidium iodide. (B) The cytotoxicity of NK-92 DsRed and NK-92 DNAM-1 DsRed against leukemia cell line (KG-1) was analyzed and compared using a cytotoxicity assay. Red line indicates NK-92 DNAM-1 DsRed and blue indicates NK-92 DsRed.(C) The cytotoxicity of NK-92 and NK-92 TIGIT KO against leukemia cell lines was analyzed and compared using the by cytotoxicity assay. Orange line indicates NK-92 TIGIT KO and blue indicates NK-92. A two-tailed Student' s t-test was used for comparison between the groups. Mean ± SE is shown. ***P < 0.001, ****P < 0.0001, E: effector, T: target.

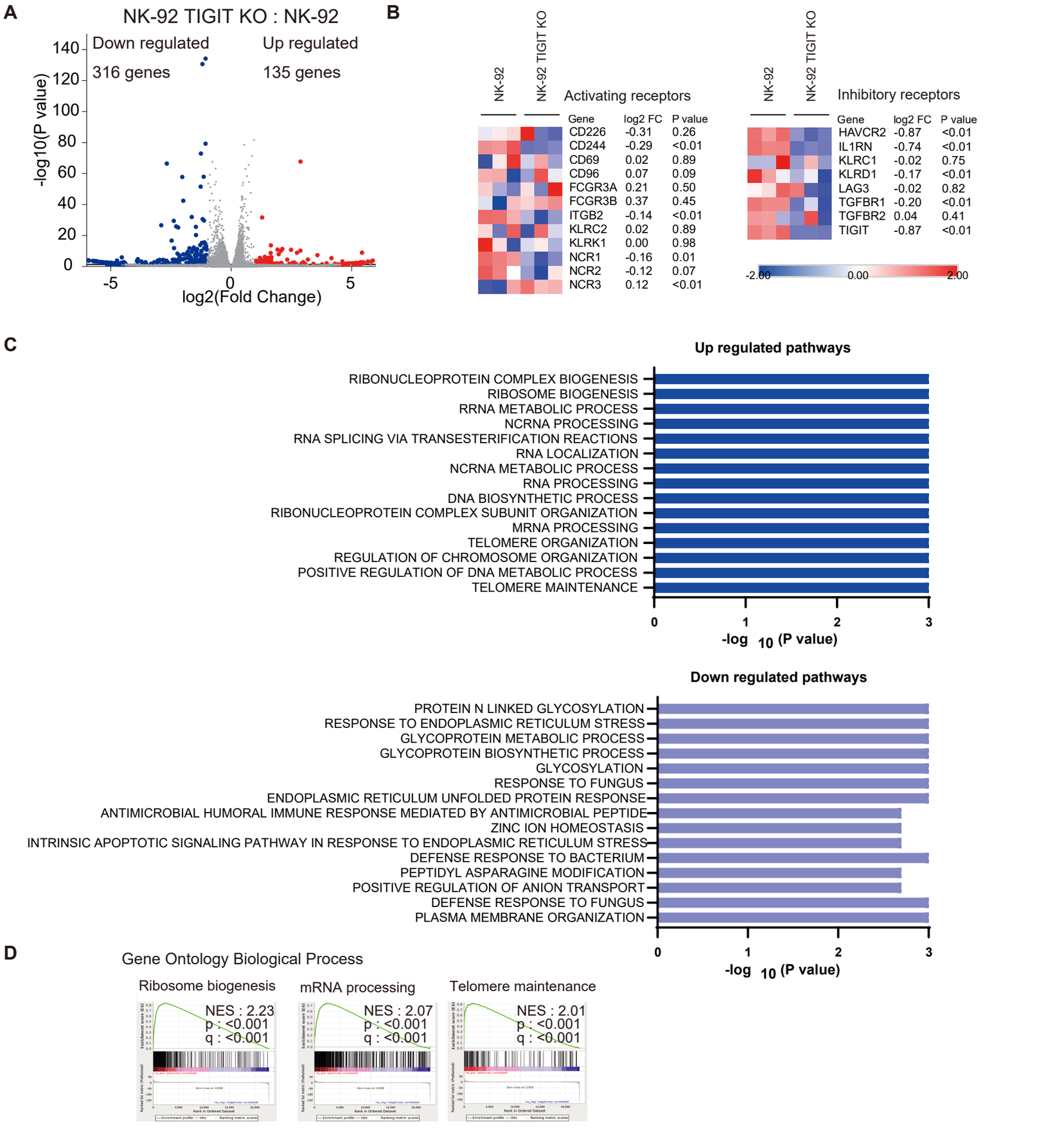


Supplementary Figure 11. Gating strategy for degranulation assay, and cytokine assay. (A) Schematic diagram of NK cell degranulation and intracellular cytokine production analysis using flow cytometry. FSC, forward scatter; SSC, side scatter; CFSE, carboxyfluorescein succinimidyl ester. (B) CD107a expression in NK-92 DsRed / NK-92 DNAM-1 DsRed was analyzed using flow cytometry when NK-92 DsRed / NK-92 DNAM-1 DsRed and leukemia cell line (KG-1) were co-cultured. TNFα/IFNγ expression in NK-92 DsRed /NK-92 DNAM-1 DsRed was analyzed using intracellular flow cytometry. (C) CD107a expression in of NK-92/NK-92 TIGIT KO was analyzed by using flow cytometry when NK-92/NK-92 TIGIT KO and leukemia cell lines were co-cultured. TNFα/IFNγ expression in of NK-92/NK-92 TIGIT KO cells were analyzed by using intracellular flow cytometry. Comparisons between the groups were performed using a two-tailed Student' s t-test. Mean ± SE is shown. ns: no significant difference. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

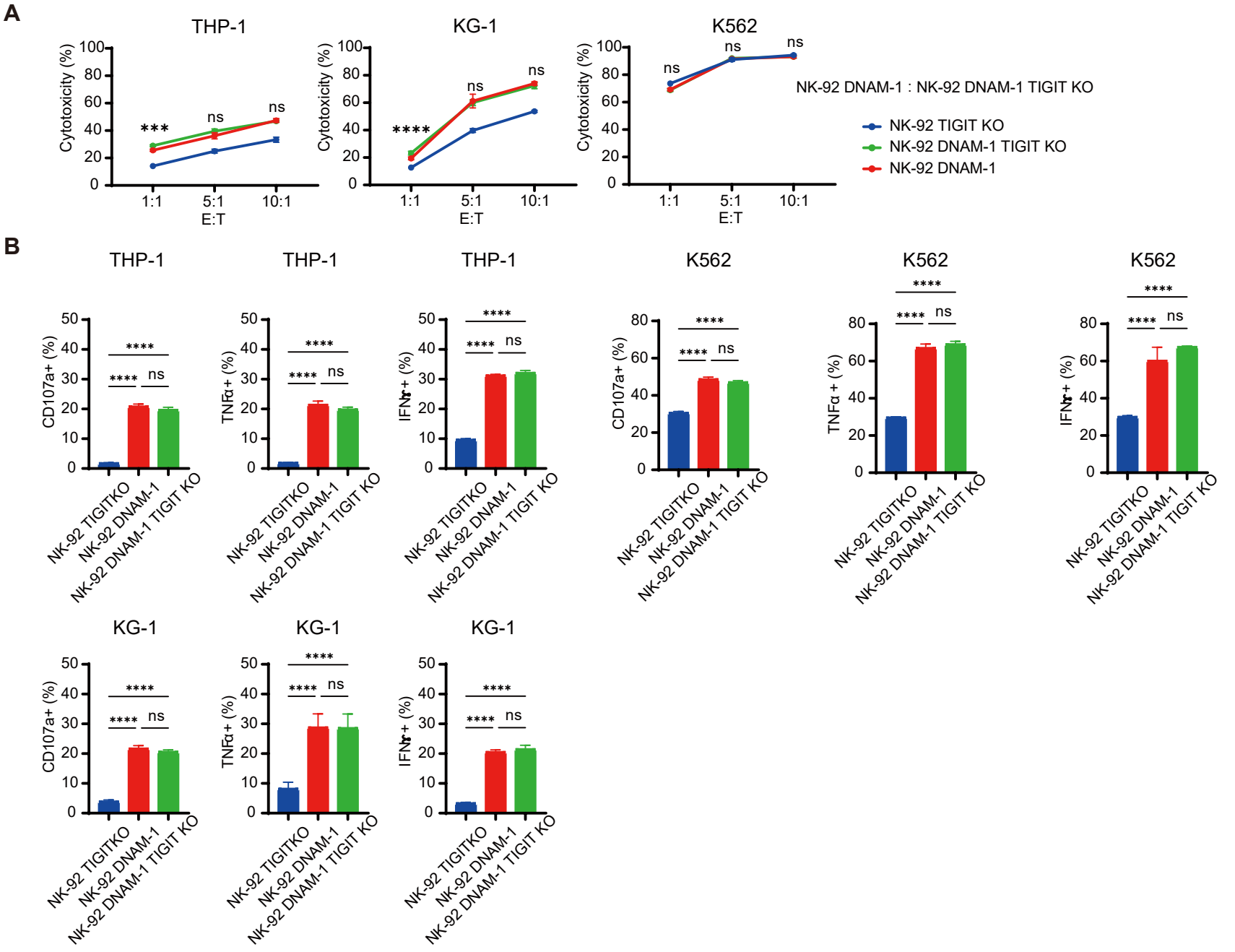




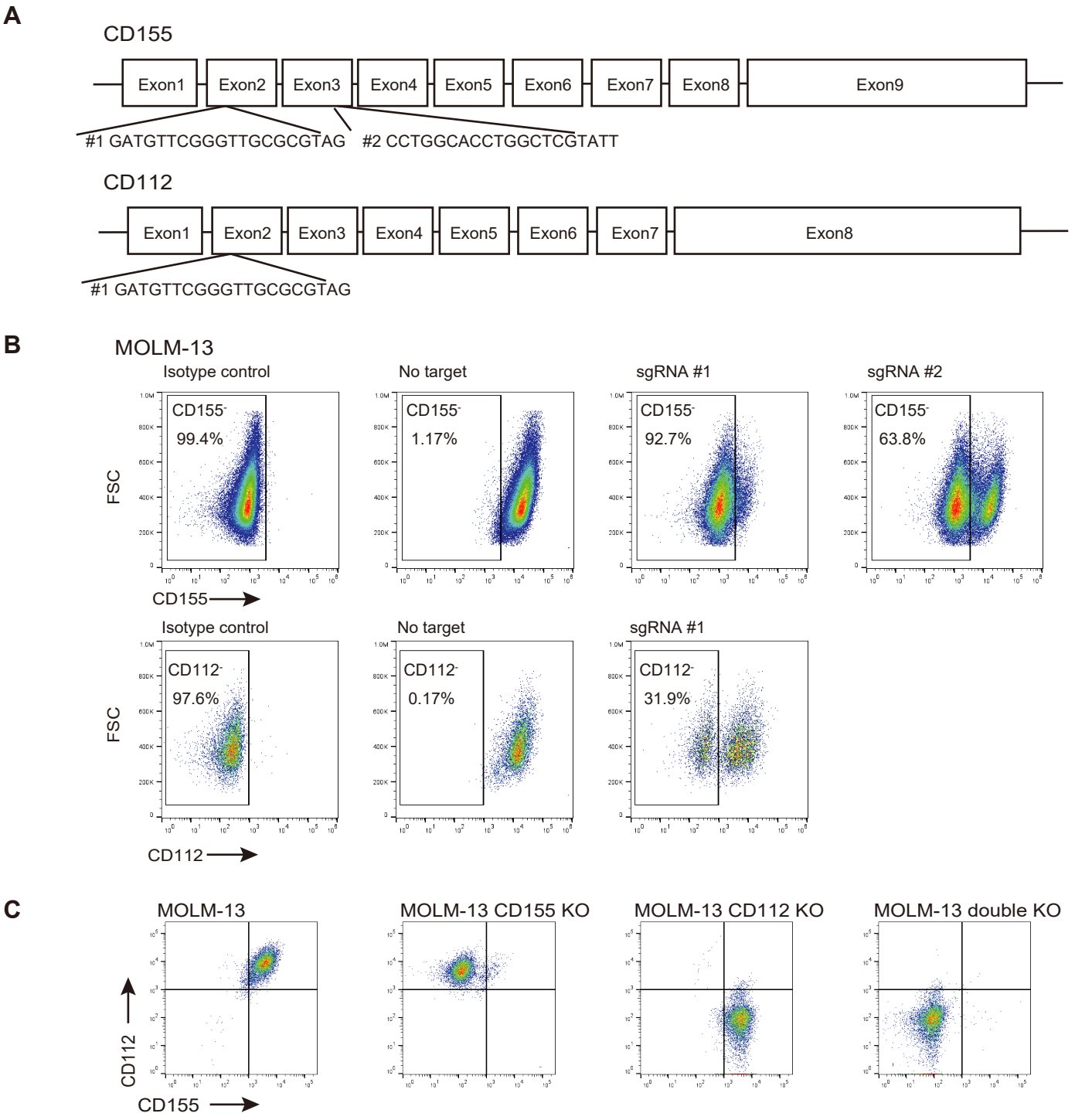
Supplementary Figure 12. NK-92 DNAM-1 DsRed showed increased expression of cytotoxicity-related genes. Gene Set Enrichment Analysis of for genes upregulated or downregulated gene pathways in NK-92 DNAM-1 DsRed compared with those in NK-92 DsRed, indicating the Gene Ontology Biological Process gene set with $P < 0.05$.



Supplementary Figure 13. NK-92 TIGIT KO decreased the expression of NK cell inhibitory receptor-related genes. (A) Volcano plot of genes with variable expression based on RNA-seq. Log2 (fold change (FC)) is plotted on the X-axis and -log10 (P value) on the Y-axis. Gray points indicate |log2 (FC)| ≤ 1 and P ≥ 0.05, red points indicate log2 (FC) ≥ 1 and P < 0.05, and blue points indicate that log2 (FC) < -1 and P < 0.05. (B) Heat map of NK cell activating receptor-related genes, and NK cell inhibitory receptor-related genes. (C) GSEA for genes in upregulated or downregulated pathways in NK-92 TIGIT KO compared with those in to NK-92, indicating a Gene Ontology Biological Process gene set with P < 0.05. (D) Representative data from the GSEA of a Gene Ontology Biological Process gene set. NES: Normalized enrichment score, q: false discovery rate.



Supplementary Figure 14. No additive effect of DNAM-1 transduction and TIGIT KO is observed. (A) Cytotoxicity of NK-92 DNAM-1, NK-92 TIGIT KO, and NK-92 DNAM-1 TIGIT KO against leukemia cell lines (THP-1, KG-1, and K562) was analyzed and compared using the cytotoxicity assay. The red, blue, and green lines indicate NK-92 DNAM-1, NK-92 TIGIT KO, and NK-92 DNAM-1 TIGIT KO, respectively. (B) CD107a expression in NK-92 DNAM-1, NK-92 TIGIT KO, and NK-92 DNAM-1 TIGIT KO cells co-cultured with leukemia cell lines was analyzed using flow cytometry. TNFα/IFNγ expression in NK-92 DNAM-1, NK-92 TIGIT KO, and NK-92 DNAM-1 TIGIT KO mice was analyzed using intracellular flow cytometry. Mean ± SE is shown, a two-tailed Student' s t-test was used for comparison between two groups, and a one-way ANOVA test was used for comparison among three groups. Multiple comparisons were performed using Tukey' s test. ns: no significant difference, ***P < 0.001, ****P < 0.0001



Supplementary Figure 15. Generation of CD155 and/or CD112 KO cells. (A) Target sequences of sgRNA used for CD155/CD112 KO and their sites. (B) Flow cytometric analysis of CD155/CD112 KO using the Crispr-Cas9 system. FSC: forward scatter (C) Representative data of AML cell line with CD155 and/or CD112 KO. Their expression was analyzed using flow cytometry.

Supplementary Table1: List of flow cytometry antibodies

Source	Specificity	Fluorochrome	Clone	Cat.
BioLegend	CD107a(LAMP-1)	APC	H4A3	328619
BioLegend	CD112(Nectin-2)	PE	TX31	337410
BioLegend	CD112(Nectin-2)	APC	TX31	337411
BioLegend	CD155(PVR)	PE	TX24	337508
BioLegend	CD226(DNAM-1)	FITC	11A8	338303
BioLegend	CD226(DNAM-1)	APC	11A8	338312
BioLegend	CD96(TACTILE)	PE/Cyamine7	NK92.39	338416
BioLegend	TIGIT(VSTM3)	PE	A15153G	372704
BioLegend	Mouse IgG1 k isotype	FITC	MOPC-21	400110
BioLegend	Mouse IgG1 k isotype	PE	MOPC-21	400112
BioLegend	Mouse IgG1 k isotype	APC	MOPC-21	400120
BioLegend	Mouse IgG1 k isotype	PE/Cyamine7	MOPC-21	400126
BioLegend	Mouse IgG1 k isotype	APC/Cyamine7	MOPC-21	400127
BioLegend	Rat IgG1	Alexa Fluor 488	RMG1-1	406626
BioLegend	Monensin Solution			420701
BioLegend	IFN γ	PE/Cyamine7	4S.B3	502527
BioLegend	TNF α	APC	MAb11	502913
R&D Systems	CD112R	Alexa Fluor 488	2334A	FAB93651G
BioLegend	CD13	BV605	WM15	301727
BioLegend	CD16	Alexa Fluor 700	3G8	302025
BioLegend	CD235ab	PE-Cy5	HIR2	306606
BioLegend	CD112	PE	TX31	337409
BioLegend	CD155	BV421	SK II .4	337631
BioLegend	CD226(DNAM-1)	BV510	11A8	338329
BioLegend	CD96(TACTILE)	BV421	NK92.39	338417
BD Biosciences	CD45	PerCP-Cy5.5	2D1	340953
BioLegend	CD3	APC/Cyamine7	SK7	344818
BioLegend	TIGIT(VSTM3)	PE/Dazzle 594	A15153G	372715
BD Biosciences	CD56	Alexa Fluor 700	B159	557919
R&D Systems	A2aR	PE	599717	FAB94971P
BECKMAN COULTER	CD34	FITC	581	IM1870

Supplementary Table 2. Characteristics of de novo AML patients

Patient no.	Age	Sex	WHO classification	Cytogenetics	Molecular biology	Samples	Blasts (%)	Sorting
1	72	M	AML, NOS	Complex	Negative	PB	4.0	Yes
						BM	41.5	-
2	71	F	AML with CEBPA mutation	Normal	*SRSF2*, *CEBPA*	BM	25.0	Yes
3	58	F	Therapy related AML	t(9;11)(p21.3;q23.3)	Negative	PB	74.5	Yes
4	68	M	AML with RUNX1-RUNX1T1	t(8;21)(q22;q22)	Negative	PB	53.5	Yes
5	35	M	AML, NOS	Normal	*c-Kit*(D816V), *MYC*	PB	98.5	Yes
6	28	F	AML, NOS	t(3;3)(q21.3;q26),-7	Negative	PB	28.0	No
7	77	F	AML with CEBPA mutation	add(11)(q13)	*CEBPA*	PB	49.5	No
8	48	M	AML with MRC	Complex	*FLT3*-TKD	PB	12.5	No
9	83	M	AML with maturation	Normal	Negative	PB	71.5	No
10	55	M	AML with maturation	add(X)(p22.1)	Negative	BM	41.8	-
11	62	M	AML with NPM1 mutation	Normal	*FLT3*-TKD, *NPM1*	PB	88.0	Yes
12	72	F	AML with MRC	Complex	Negative	PB	53.0	No
13	61	M	AML	Complex	Negative	PB	91.5	No
						BM	41.8	-
14	61	F	AML with maturation	+8	*FLT3*-ITD	PB	5.5	No
						BM	31.8	-
15	78	M	Therapy related AML	Complex	Negative	PB	60.0	Yes
						BM	-	-
16	55	F	AML with maturation	Normal	*IDH2*	PB	0.0	No
						BM	30.2	-
17	35	F	AML with maturation	t(8;21)(q22;q22), -X	Negative	PB	28.5	Yes
18	70	F	AML, NOS	Normal	N/A	PB	88.0	No
19	72	M	AML with MRC	+8, +13	Negative	BM	13.0	-
20	66	M	AML with maturation	Complex	Negative	PB	93.5	Yes
21	69	M	AML with MRC	Complex	Negative	PB	25.5	No
22	68	M	AML with maturation	t(8;14)(q11.2:p13)	Negative	PB	6.0	Yes
						BM	37.8	-
23	70	M	AML with maturation	-Y, del(20)(q11.2q13.3)	Negative	BM	24.0	-
24	62	F	AML with RUNX1-RUNX1T1	t(8;21)(q22;q22), del(9)(q?)	Negative	PB	37.0	No
25	69	M	AML, NOS	Normal	*FLT3*-ITD	PB	82.0	Yes
26	75	M	AML with MRC	Normal	*FLT3*-ITD	PB	95.5	Yes
27	77	F	AML with maturation	+8	Negative	PB	32.0	No
28	71	F	AML with maturation	Normal	Negative	PB	37.0	Yes
29	42	M	AML with RUNX1-RUNX1T1	t(8;21)(q22;q22), -Y	*c-Kit*(D816H)	PB	45.5	Yes
						BM	60.2	-
30	61	M	AML with maturation	N/A	Negative	PB	33.5	Yes

Note
F: Female, M: Male, AML: acute myeloid leukemia, NOS: not otherwise specified, MRC: myelodysplasia-related changes
SRSF2: serine/arginine-rich splicing factor 2, CEBPA: CCAAT/enhancer-binding protein alpha, KIT: KIT proto-oncogene receptor tyrosine kinase, MYC: MYC proto-oncogene, bHLH transcription factor, FLT3: FMS-like Tyrosine Kinase 3, ITD: internal tandem duplication mutation, TKD: tyrosine kinase domain mutation, NPM1: Nucleophosmin 1
PB: Peripheral blood, BM: Bone marrow, N/A: not available, Sorting Yes: sorting and used in cytotoxicity assay, No: not sorting