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

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

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



## Abstract

Acute myeloid leukemia (AML) is a hematologic malignancy that frequently relapses, even if remission can be achieved with intensive chemotherapy. One known relapse mechanism is the escape of leukemic cells from immune surveillance. Currently, there is no effective immunotherapy for AML because of the lack of specific antigens. Here, we aimed to elucidate the association between CD155 and CD112 in AML cell lines and primary AML samples and determine the therapeutic response. Briefly, we generated NK-92 cell lines (NK-92) with modified DNAX-associated molecule 1 (DNAM-1) and T-cell immunoglobulin and ITIM domain (TIGIT), which are receptors of CD155 and CD112, respectively. Analysis of 200 cases of AML indicated that the survival of patients with high expression of CD112 was shorter than that of patients with low expression. NK-92 DNAM-1 exhibited enhanced cytotoxic activity against AML cell lines and primary cells derived from patients with AML. DNAM-1 induction in NK-92 cells enhanced the expression of cytotoxicity-related genes, thus overcoming the inhibitory activity of TIGIT. Between CD155 and CD112, CD112 is an especially important target for natural killer (NK)-cell therapy of AML. Using a xenograft model, we confirmed the enhanced antitumor effect of NK-92 DNAM-1 compared with that of NK-92 alone. We also discovered that CD112 (Nectin-2), an immune checkpoint molecule belonging to the Nectin/Nectin-like family, functions as a novel target of immunotherapy. In conclusion, modification of the DNAM-1/CD112 axis in NK cells may be an effective novel immunotherapy for AML. Furthermore, our findings suggest that the levels of expression of these molecules are potential prognostic markers in AML.

## Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy in which hematopoietic stem/progenitor cells accumulate multiple genetic abnormalities associated with differentiation and proliferation, resulting in the autonomous and uncontrolled progression of the disease.[1,2] Recently, molecular targeted agents such as venetoclax[3] and gilteritinib[4] have been approved for use as AML treatment. However, AML relapse remains a major concern and is associated with a poor prognosis.[5]
Escape of tumor cells from the immune system has piqued interest as an important mechanism of relapse in hematologic malignancies. Previous studies have documented enhanced expression of inhibitory receptors such as programmed death-1 in T cells derived from patients with AML.[6,7] To circumvent escape from the immune system, immune checkpoint inhibitors have been tested for AML treatment, but they have not been effective.[8,9] Moreover, the development of chimeric antigen receptor T-cell therapy for AML has stalled because of the lack of specific targetable antigens.[10]
We focused on natural killer (NK) cells, a type of innate lymphocyte capable of directly killing target cells without antigen presentation.[11] The cytotoxic function of NK cells is regulated by the balance of signals from activating and inhibitory receptors.[12]
NK-cell therapy for AML includes autologous and allogeneic

NK cells. Autologous NK cells are less likely to cause graft-*versus*-host disease, but they do not exhibit substantial therapeutic efficacy because of their own reduced cytotoxicity.[13,14] Difficulties with *in vitro* expansion and activation, and lack of *in vivo* persistence of allogeneic NK cells contribute to insufficient clinical efficacy.[15]

The NK-92 cell line (NK-92), which is derived from a patient with non-Hodgkin lymphoma, has been employed in off-the-shelf therapy,[16,17] as it has just one receptor of the inhibitory killer cell immunoglobulin-like receptor (KIR) family members,[18] expresses many activating receptors,[19] and is more cytotoxic than primary NK cells. A clinical trial using NK-92 for relapsed/refractory AML was conducted, but the results were not promising.[20]

Recently, the pathway between immune cell receptors, including DNAX-associated molecule 1 (DNAM-1, CD226), T-cell immunoglobulin and ITIM domain (TIGIT), PVR-related Ig domain (PVRIG, CD112R), T-cell activation, increased late expression (TACTILE, CD96), and their ligands, the Nectin/Nectin-like family molecules CD155 (poliovirus receptor), CD111 (Nectin-1), CD112 (Nectin-2), and CD113 (Nectin-3), have attracted attention as a novel class of immune checkpoint molecules. The major receptors in this pathway are thought to be DNAM-1 (an activating receptor) and TIGIT (an inhibitory receptor).[21,22] CD155 and CD112 belong to the immunoglobulin superfamily of adhesion glycoproteins[23] and their expression is upregulated in a variety of malignancies, although their association with prognosis varies by the type of malignancy.[24-26] There are reports of decreased DNAM-1 expression in NK cells and increased TIGIT expression in exhausted T cells derived from AML.[27-30] TIGIT antibodies have been shown to be useful in the treatment of solid tumors and are being tested in clinical trials, but have not been shown to be useful in AML.[31,32]

In this study, we analyzed the expression of CD155 and CD112 in AML cell lines and primary AML samples to elucidate their association with therapeutic responses. We found that CD112 may be associated with a poor prognosis. Furthermore, we established a DNAM-1-introduced NK-92 assuming that CD112 targeted therapy may be more promising than previous immunotherapies for patients with a poor prognosis.

# Methods

### Analysis using a clinical database

The Cancer Genome Atlas (TCGA) program (*https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga*) was used to analyze data in the cBioPortal for Cancer Genomics (version 5.1.2) (*https://www.cbioportal.org/*).[33] mRNA expression in TCGA dataset was assigned to the group with a threshold Z-score >1.0. Kaplan-Meier overall survival curves were analyzed using the log-rank test.

### Cytotoxicity assay

Leukemia cell lines (primary AML cells) were stained with carboxyfluorescein succinimidyl ester (CellStain-CFSE; Dojindo, Kumamoto, Japan). Tumor cells (target cells) and NK-92/modified NK-92 (effector cells) were co-cultured for 4 h at effector-to-target cell ratios of 1, 5, and 10. Four hours later, the cells were collected and stained with Cellstain-propidium iodide (PI) solution (Dojindo). The percentage of PI-positive leukemia cell lines cultured alone or co-cultured with leukemia cell lines was estimated using flow cytometry. Cytotoxicity of NK-92 and modified-NK-92 was calculated as the percentage of PI-positive co-cultured leukemia cell lines minus that of leukemia cell lines cultured alone.

### Primary cells

Primary cells were stained with the antibodies listed in *Online Supplementary Table S1*. The cells were analyzed and isolated using BD FACS Aria II SORP (BD Biosciences). FlowJo software (version 10.8.0, FlowJo LLC, Ashland, OR, USA) was used to analyze the results. This study was reviewed and approved by the Ethics Review Committee of the Institute of Medical Science, University of Tokyo (2020-16-0623).

### Leukemia xenograft models

Female (6- to 8-week-old) NOD.Cg-Prkdc$^{scid}$ Il2rg$^{tm1Wjl}$/ SzJ (NSG) mice were irradiated with 200 cGy on day -1. Luciferase-introduced MOLM-13 and phosphate-buffered saline (PBS) (FUJIFILM Wako, Japan)/NK-92/NK-92 DNAM-1 cells were injected through the tail vein on day 0. PBS/NK-92/NK-92 DNAM-1 cells were injected through the tail vein on days 1 and 2. D-luciferin (FUJIFILM Wako Pure Chemicals, Osaka, Japan) was administered intraperitoneally at a dose of 100 μL (150 μg) to each animal twice a week and evaluated using the Spectrum In Vivo Imaging System (IVIS, PerkinElmer, Waltham MA, USA). Living Image Software (version 4.7.4, PerkinElmer) was used to quantify the luminescence. Details on these protocols are included in the *Online Supplementary Methods*. Additional information on the methods is provided in the *Online Supplementary Data*.

# Results

### High CD112 expression is associated with a poor prognosis in patients with acute myeloid leukemia

To confirm the association between Nectin/Nectin-like family molecule expression and prognosis in AML patients, we analyzed data from patients with AML using cBioPortal. We analyzed the AML cohort of TCGA program and found no significant difference in survival rate between the groups with high or low CD155 ($P$=0.972). However, the CD112 high-expression group had a significantly worse prognosis than the low-expression group ($P$=0.014) (Figure 1A).

Analysis of TCGA clinical data showed an increased frequency of patients with adverse-risk cytogenetics according to the European LeukemiaNet (ELN) risk classification in the

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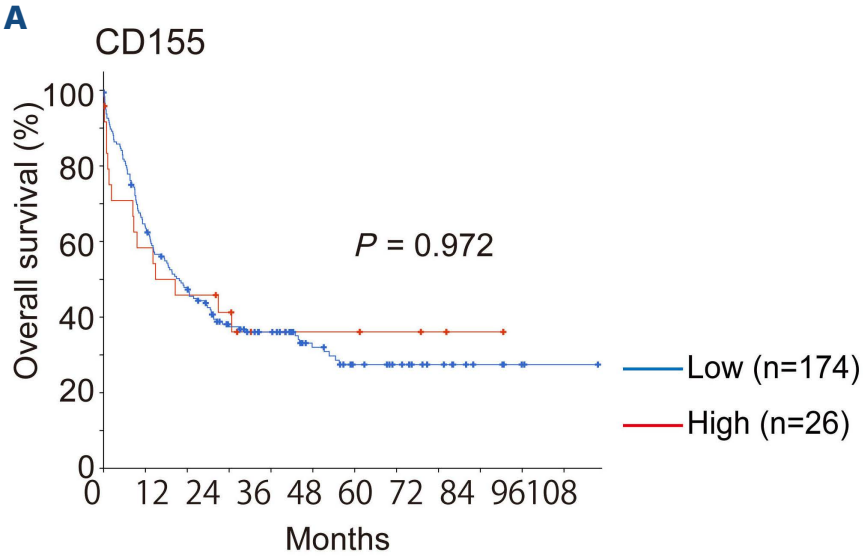


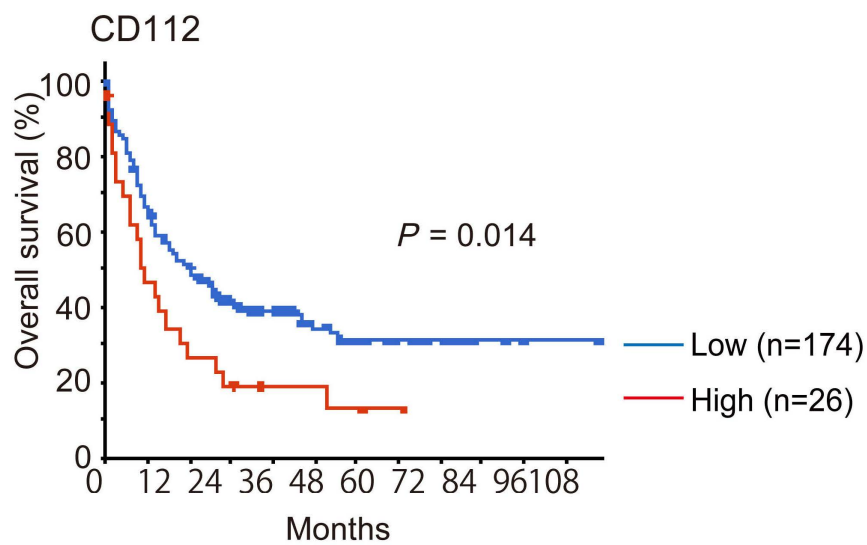


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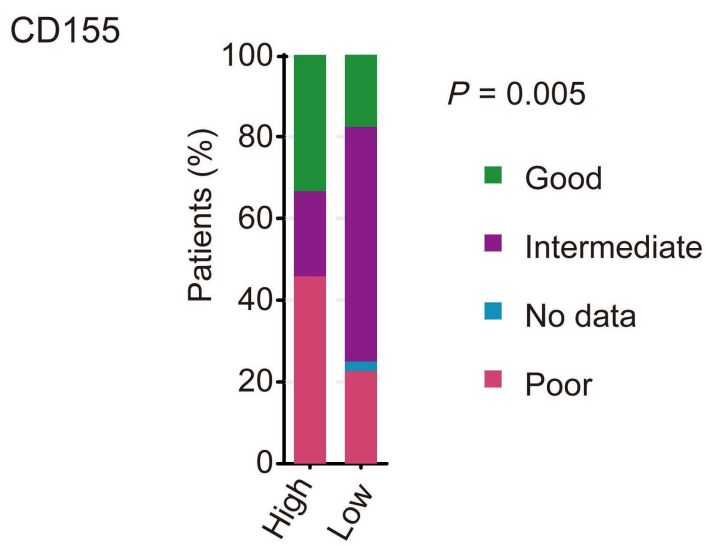


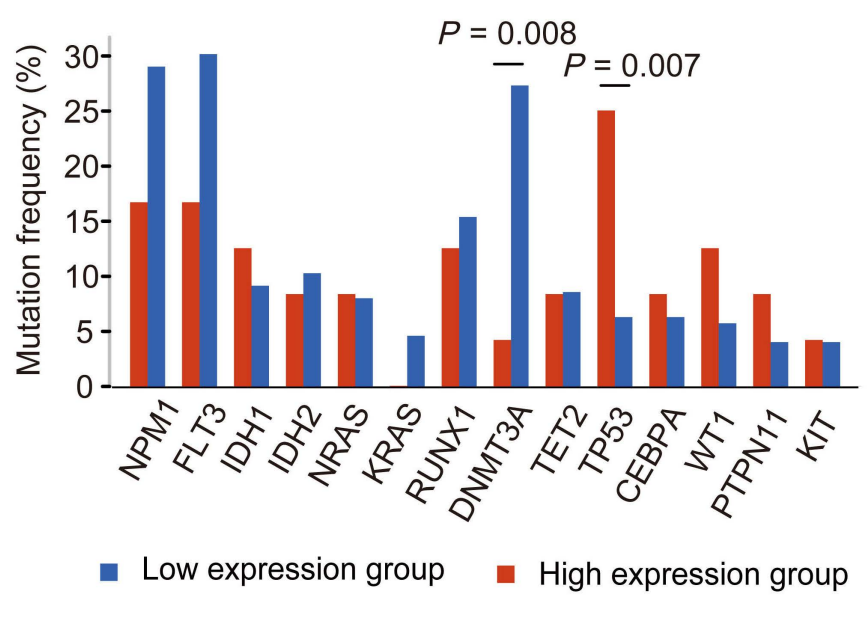


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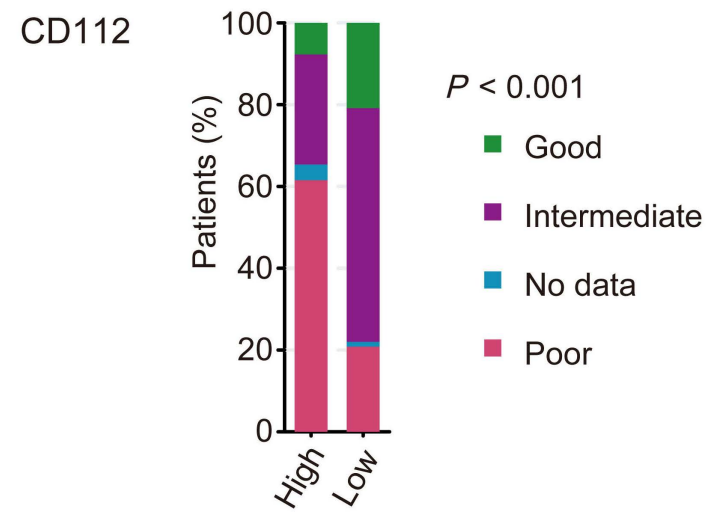


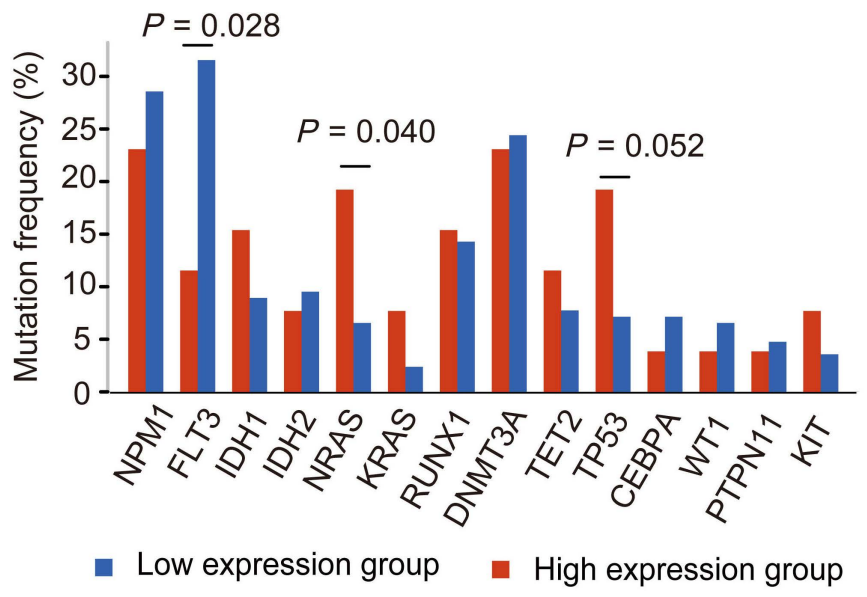


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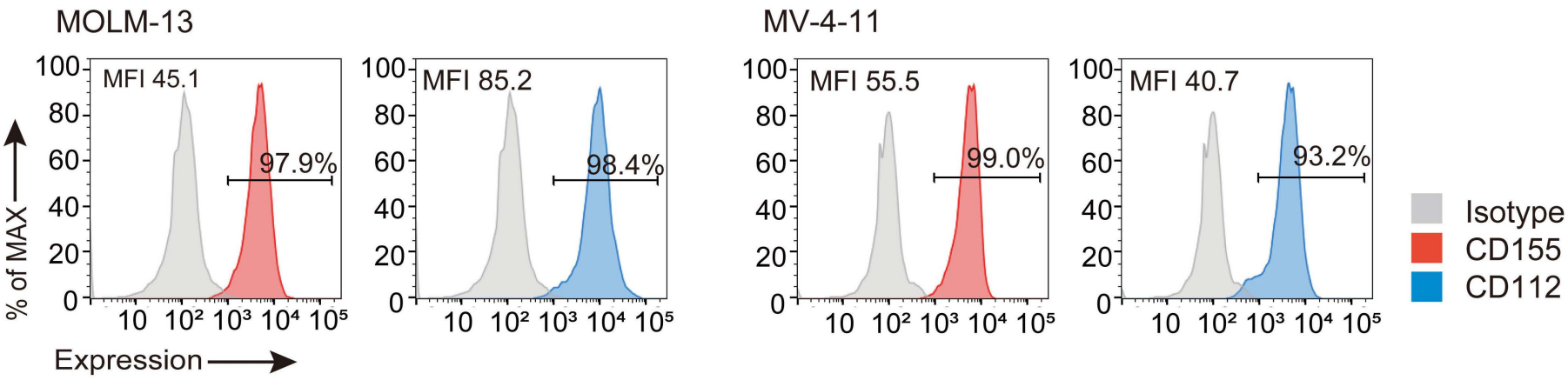


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**Figure 1. Relationship between CD155 and CD112 expression and prognosis in patients with acute myeloid leukemia.** (A) The cBioPortal was used to analyze the association between CD155 and CD112 expression and prognosis in a cohort of patients with acute myeloid leukemia (AML) from The Cancer Genome Atlas (TCGA). A log-rank test was used. The red line indicates the high expression group and the blue line represents the low expression group. (B, C) Left: relationship between CD155/CD112 expression and European LeukemiaNet risk classification in a cohort of patients with AML derived from TCGA. The difference in the distribution of patients with each cytogenetic risk between the high and low expression groups was estimated using the $\chi^2$ test. Right: relationship between CD155/CD112 expression and genetic mutation in a cohort of patients with AML derived from TCGA. Red indicates the high expression group and blue indicates the low expression group. (D) Expression of CD155 and CD112 in AML cell lines (MOLM-13 and MV-4-11) was analyzed using flow cytometry. Gray histograms indicate the isotype control, red indicates CD155 and blue indicates CD112 expression. MFI: median fluorescence intensity.

CD112 high-expression group compared with that in the low-expression group ($P$<0.001). In a multivariate analysis, CD112 was not identified as an independent prognostic factor for overall survival. We could not find any consistent correlation between CD155 expression and ELN risk classification ($P$=0.005) (*Online Supplementary Figure S1A*). Regarding somatic mutations, the CD112 high-expression group had a significantly lower frequency of *FLT3* mutations and significantly higher frequency of *NRAS* mutations than the low-expression group. Although the difference was not statistically significant, *TP53* mutations were also frequently found in the CD112 high-expression group. Other clinical data did not differ significantly between CD155 or CD112 high- and low-expression groups (Figure 1B, C, *Online Supplementary Figure S1A, B*).

## Leukemia cell lines express CD155/CD112 on the cell surface

The expression of CD155 and CD112 in AML cell lines (MOLM-13, MV-4-11, THP-1, KG-1, NB-4, Kasumi-1, TF-1) and a blastic phase of a chronic myeloid leukemia cell line (K562) was analyzed using flow cytometry. Both CD155 and CD112 were expressed in both types of cell line, and more than 90% of the cells expressed these molecules, except for CD112 in NB-4 (Figure 1D, *Online Supplementary Figure S2*).

## CD155 and CD112 are expressed in primary acute myeloid leukemia blasts and their expression is not associated with the initial treatment response

To analyze the expression of Nectin/Nectin-like family molecules and their receptors in leukemia cells from patients with AML, we analyzed the expression of CD155 and CD112 in primary AML blasts from patients with *de novo* AML using flow cytometry (*Online Supplementary Figure S3*). The patients' characteristics are shown in *Online Supplementary Table S2*. As for primary AML blasts in peripheral blood, 63.0% (17/27 cases) had more than 20% positivity for both CD155 and CD112 expression (Figure 2A).

Next, we analyzed the relationship between the expression of CD155/CD112 in AML blasts and their response to therapy. We divided patients into two groups: those who achieved a complete response with standard induction therapy and those who did not and compared the expression of CD155/CD112 in AML blasts in the two groups. The expression of CD155 and CD112 in primary AML blasts did not differ significantly between the groups. Although the median CD155 and CD112 values were higher in the group that did not achieve a complete response than in the group that did, the difference in distribution was not statistically significant (*Online Supplementary Figure S4*).

Thus, AML blasts express CD155 and CD112. Although the mRNA expression of CD112 in AML blasts is related to overall survival, CD155 and CD112 expression in AML blasts was not associated with the achievement of complete response to induction therapy.

## No association between DNAM-1 or TIGIT expression on natural killer cells and initial treatment response in patients with acute myeloid leukemia

We analyzed the expression of DNAM-1 and TIGIT in primary NK and T cells from patients with *de novo* AML using flow cytometry (*Online Supplementary Figure S3*). More than 90% of NK cells in the peripheral blood and bone marrow were positive for DNAM-1 and TIGIT. No correlation was observed between DNAM-1 and TIGIT expression (Figure 2B). All T cells in peripheral blood and bone marrow were positive for DNAM-1 and TIGIT. There was a significant positive correlation between DNAM-1 and TIGIT expression in peripheral blood T cells (*Online Supplementary Figure S5A*).

Next, we analyzed the relationship between the expression of DNAM-1 and TIGIT in NK cells and response to therapy. We divided the patients into two groups and compared the expression of DNAM-1/TIGIT in NK cells from the two groups. DNAM-1 and TIGIT expression in NK cells did not differ significantly between the groups. Regarding peripheral blood, the group that did not achieve a complete response had a lower median DNAM-1 and higher median TIGIT expression than the group that did have a complete response (*Online Supplementary Figure S5B*). In T cells, DNAM-1 and TIGIT expression did not differ significantly between the groups (*Online Supplementary Figure S5C*). No correlation was found between the clinical test data and expression of Nectin/Nectin-like family molecules or their receptors (*Online Supplementary Figure S6*).

The expression of receptors for Nectin/Nectin-like family molecules in NK-92 was analyzed using flow cytometry. TIGIT and TACTILE were expressed in more than 80% of the cells, whereas DNAM-1 was expressed in only approximately 8% of the cells, and PVRIG was almost not expressed (Figure 2C).

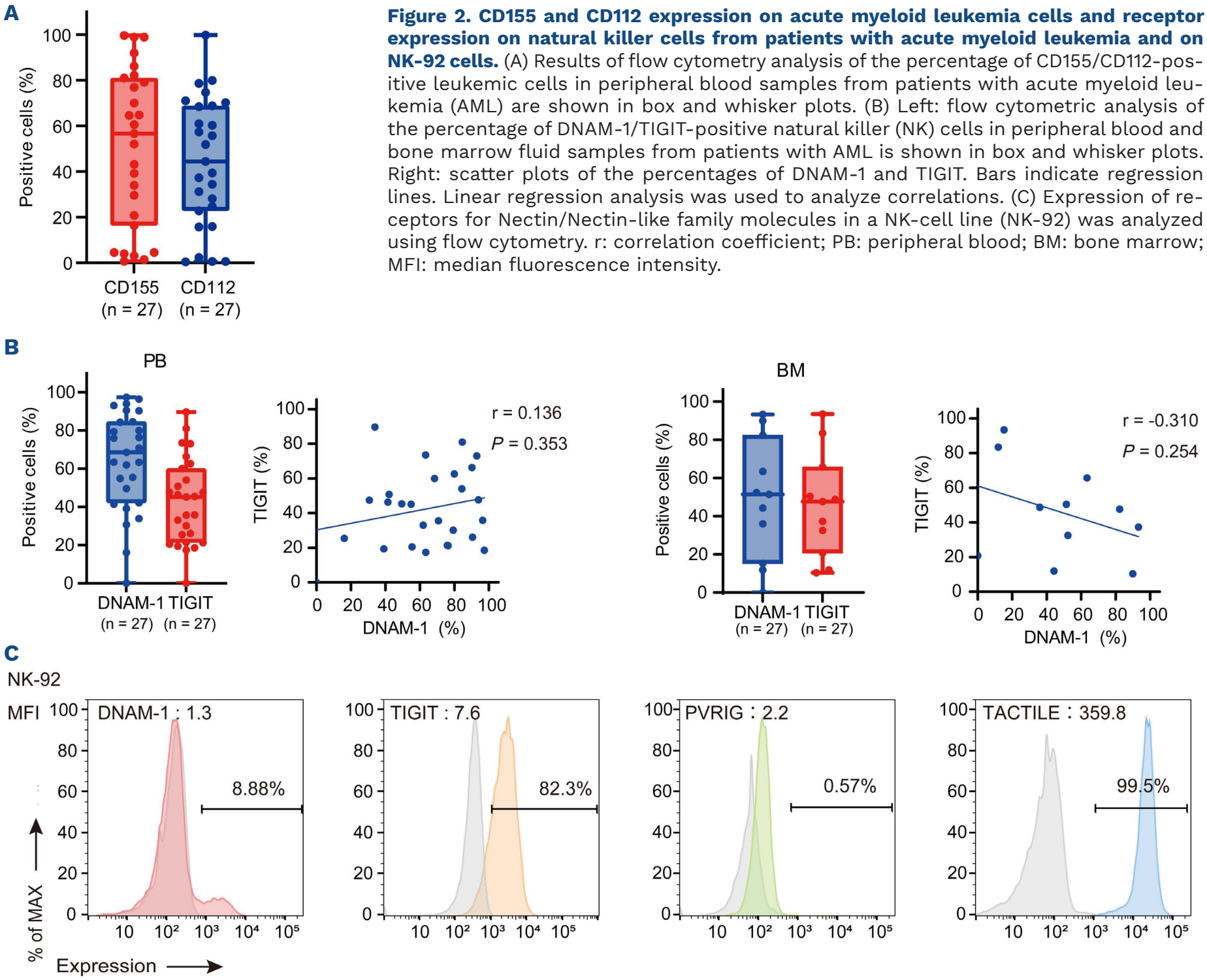


**Figure 2. CD155 and CD112 expression on acute myeloid leukemia cells and receptor expression on natural killer cells from patients with acute myeloid leukemia and on NK-92 cells.** (A) Results of flow cytometry analysis of the percentage of CD155/CD112-positive leukemic cells in peripheral blood samples from patients with acute myeloid leukemia (AML) are shown in box and whisker plots. (B) Left: flow cytometric analysis of the percentage of DNAM-1/TIGIT-positive natural killer (NK) cells in peripheral blood and bone marrow fluid samples from patients with AML is shown in box and whisker plots. Right: scatter plots of the percentages of DNAM-1 and TIGIT. Bars indicate regression lines. Linear regression analysis was used to analyze correlations. (C) Expression of receptors for Nectin/Nectin-like family molecules in a NK-cell line (NK-92) was analyzed using flow cytometry. r: correlation coefficient; PB: peripheral blood; BM: bone marrow; MFI: median fluorescence intensity.

### Nectin/Nectin-like family molecule receptor-modified NK-92 showed enhanced cytotoxicity against leukemia cell lines

To analyze the relationship between the receptors of Nectin/Nectin-like family molecules and NK-92 activity, we generated a receptor-modified NK-92. DNAM-1 complementary DNA was transfected into the cells using lentiviral particles and analyzed using flow cytometry after 1 week. Lentiviral vectors with DsRed as a marker were generated and transfected into NK-92 to create control cells. (*Online Supplementary Figure S7A, B*). The cells that were strongly positive for DNAM-1 were sorted and expanded for another week, and DNAM-1 expression was analyzed using flow cytometry. DNAM-1-induced NK-92 (NK-92 DNAM-1 DsRed) with a significantly higher mean fluorescence intensity and a positivity rate of more than 90% were obtained (Figure 3A). Electroporation was used to introduce sgRNA for knockout (KO) of TIGIT and Cas9 protein into NK-92 and analyzed using flow cytometry after 1 week (*Online Supplementary Figure S7C, D*). TIGIT-negative cells were sorted and their expression was analyzed using flow cytometry after 1 week. TIGIT-negative NK-92 (NK-92 TIGIT KO) with a significantly lower mean fluorescence intensity and a positivity rate of less than 1% were obtained (*Online Supplementary Figure S8*).

To analyze the function of DNAM-1-induced and TIGIT KO NK-92, we conducted MTT and cytotoxicity assays. Analysis of cell proliferation at 24 and 48 h indicated no significant changes in either NK-92 DNAM-1 or NK-92 TIGIT KO, compared with that in NK-92 (*Online Supplementary Figure S9*). Next, we conducted a cytotoxicity assay targeting leukemia cell lines (MOLM-13, MV-4-11, KG-1) and measured the percentage of death (PI positivity) of leukemia cells (CFSE positive) after 4 h of co-culture with NK-92 DsRed or NK-92 DNAM-1 DsRed (*Online Supplementary Figure S10A*). Both cell lines showed significantly enhanced NK-92 DNAM-1 DsRed cytotoxicity compared to NK-92 DsRed (Figure 3B,

*Online Supplementary Figure S10B*). Similar effects were confirmed in NK-92 and NK-92 TIGIT KO (*Online Supplementary Figure S10C*).

### Strong expression of DNAM-1 induces NK-92 activation and cytokine production

To evaluate DNAM-1-induced NK-cell activation, the expression of the degranulation marker (CD107a) and intracellular cytokines (tumor necrosis factor-α [TNFα] and interferon-γ [IFNγ]) of NK cells co-cultured with leukemia cell lines was analyzed using flow cytometry (*Online Supplementary Figure S11A*). Both in the absence of target cells and when co-cultured with leukemia cell lines, the expression of CD107a, TNFα, and IFNγ was significantly upregulated in NK-92 DNAM-1 DsRed, compared with that in NK-92 DsRed (Figure 3C, *Online Supplementary Figure S11B*). In contrast,

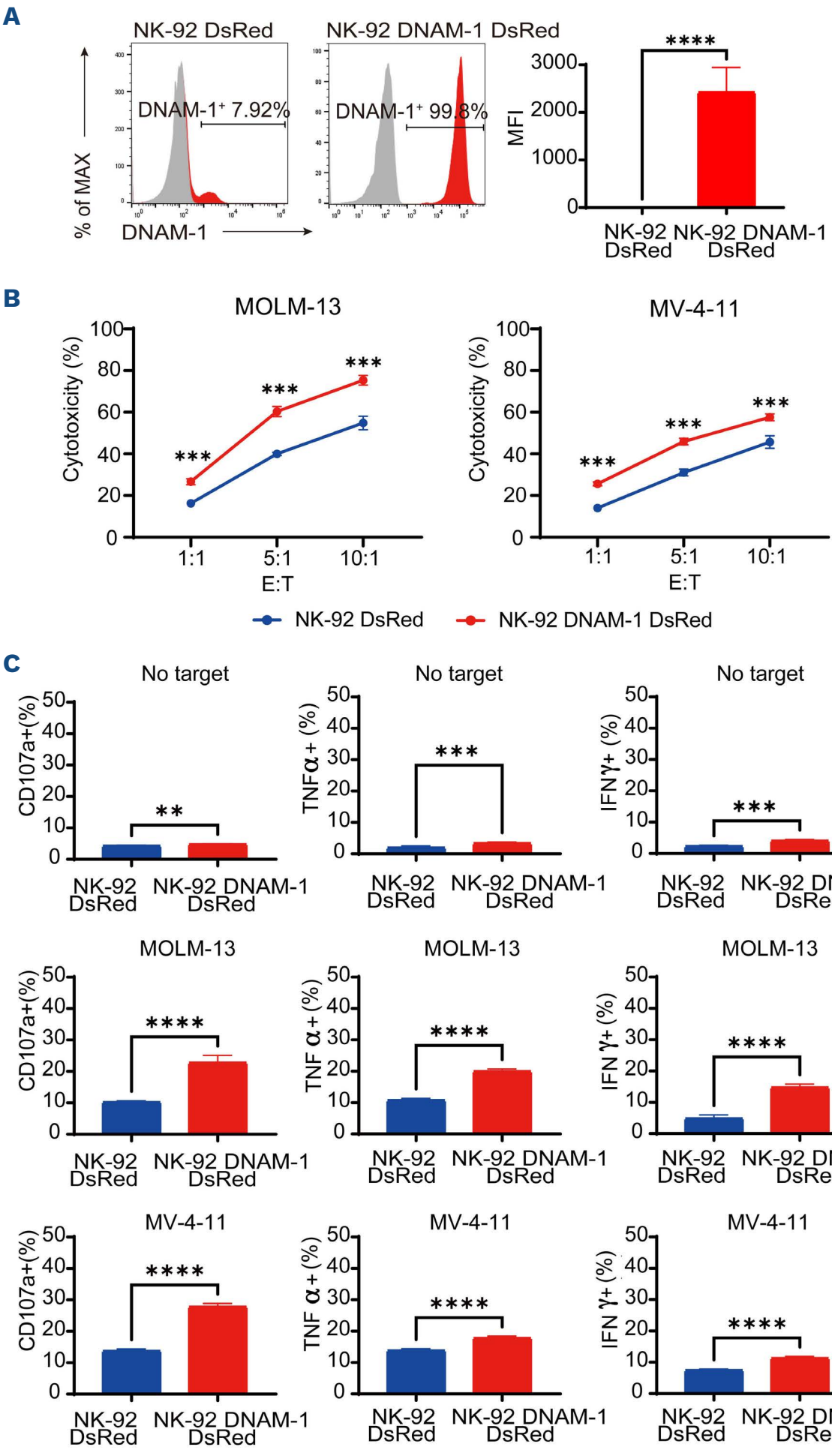


**Figure 3. Generation and functional analysis of NK-92 with modified receptors for Nectin/Nectin-like family molecules.** (A) DNAM-1 expression in NK-92 DsRed and NK-92 DNAM-1 DsRed. Gray histograms represent the isotype control, while the red histograms represent DNAM-1 expression. (B) Cytotoxicity of NK-92 DsRed and NK-92 DNAM-1 DsRed against leukemia cell lines (MOLM-13, MV-4-11) was analyzed and compared using cytotoxicity assays. Red lines indicate NK-92 DNAM-1 DsRed and blue lines indicate NK-92 DsRed. (C) CD107a expression in NK-92 DsRed/NK-92 DNAM-1 DsRed was analyzed using flow cytometry when NK-92 DsRed and NK-92 DNAM-1 DsRed and leukemia cell lines were co-cultured. Tumor necrosis factor-α and interferon-γ expression in NK-92 DsRed and NK-92 DNAM-1 DsRed was analyzed using intracellular flow cytometry. Comparisons between the groups were performed using a two-tailed Student *t* test. The mean ± standard error are shown. ***P*<0.01, ****P*<0.001, *****P*<0.0001, E: effector; T: target; MFI: median fluorescence intensity.

in the absence of target cells, the expression of CD107a, TNFα, and IFNγ was not significantly altered in NK-92 TIGIT KO compared with that in NK-92. When NK-92 TIGIT KO or NK-92 were co-cultured with target cells, increases in TNFα or IFNγ expression were observed in some cell lines; however, CD107a expression did not change significantly (*Online Supplementary Figure S11C*).

### Upregulation of cytotoxicity-related genes in NK-92 by DNAM-1 induction

RNA sequencing was performed to analyze the genes with changes in expression due to DNAM-1 induction or TIGIT KO. We identified genes that showed significant expression changes (|log2 (fold change)| ≥1 and $P$<0.05) after DNAM-1 induction. The expression of 128 genes, including those related to heat shock protein (HSP), interleukin and KIR, was upregulated. The expression of 110 genes containing those related to T-cell function was downregulated (Figure 4A, B). Based on previous reports,[34,35] we analyzed activating and inhibitory receptor-related genes. The expression of *CD244, FCGR3B, HAVCR2*, and others was significantly altered, but less than 2-fold (Figure 4C).

Gene set enrichment analysis showed that pathways such as Regulation of NK cell mediated immunity, Positive regulation of cell killing, and Positive regulation of cytokine production involved in immune response were significantly upregulated in the Gene Ontology Biological Process gene set (Figure 4D, *Online Supplementary Figure S12*). None of the downregulated genes, such as those related to amino acid transport, was associated with cell killing or antitumor effects.

In contrast, among the genes that showed more than 2-fold and significant changes of expression by KO of TIGIT, 135 genes were upregulated and 316 genes were downregulated (*Online Supplementary Figure S13A*). Several inhibitory receptor-related genes (*HAVCR2, IL1RN, KLRD1,* and *TGFBR1*) were significantly downregulated (*Online Supplementary Figure S13B*). The gene set enrichment analysis showed upregulation of genes related to ribosome biogenesis, mRNA processing, and telomere maintenance in the Gene Ontology Biological Process gene set (*Online Supplementary Figure S13C, D*).

Thus, strong DNAM-1 expression upregulates the expression of cytotoxicity-related genes in NK-92; however, TIGIT KO did not induce upregulation of cytotoxicity-related genes in NK-92.

### NK-92 DNAM-1 does not show any additive effect of the cytotoxicity caused by TIGIT KO.

From previous analyses, the activation of NK-92 was more evident under DNAM-1 induction than under TIGIT KO. To evaluate whether there is an additive effect of TIGIT KO on NK-92 activation by DNAM-1 induction, we generated NK-92 transfected with DNAM-1 and TIGIT KO (NK-92 DNAM-1 TIGIT KO) (Figure 5A).

Cytotoxicity assays targeting leukemia cell lines were performed using NK-92 DNAM-1, NK-92 TIGIT KO, and NK-92 DNAM-1 TIGIT KO. NK-92 DNAM-1 showed significantly increased cytotoxicity, compared to NK-92 TIGIT KO, but not when compared to K562 (Figure 5B, *Online Supplementary Figure S14A*). In K562, the cytotoxicity of NK-92 TIGIT KO was very strong, even at an effector-to-target ratio of 1:1, and there was no significant difference in cytotoxicity between NK-92 DNAM-1 and NK-92 TIGIT KO. Furthermore, no significant difference was observed between NK-92 DNAM-1 and NK-92 DNAM-1 TIGIT KO.

Next, using flow cytometry, we analyzed the expression of CD107a, TNFα, and IFNγ in NK-92 co-cultured or not with leukemia cell lines. In NK-92 DNAM-1 or NK-92 DNAM-1 TIGIT KO, CD107a, TNFα, and IFNγ expression was significantly upregulated compared with that in NK-92 TIGIT, with or without co-culture with target cells. However, no significant difference was observed between NK-92 DNAM-1 and NK-92 DNAM-1 TIGIT KO (Figure 5C, *Online Supplementary Figure S14B*).

### Enhancement of cytotoxicity by DNAM-1 induction is ligand-dependent

To analyze whether the enhancement of cytotoxicity by DNAM-1 induction was dependent on CD155 and CD112, we generated CD155 and/or CD112 KO cells in some leukemia cell lines (*Online Supplementary Figure S15A-C*).

Cytotoxicity assays were performed using NK-92 DNAM-1; the cytotoxicity of NK-92 DNAM-1 was compared between wild-type and CD155 KO, CD112 KO, or double KO cell lines. When targeting MOLM-13, single KO of CD155 or CD112 did not reduce cytotoxicity of NK-92 DNAM-1, but double KO significantly reduced its cytotoxicity compared to the wild-type. When targeting MV-4-11, CD155 KO did not reduce cytotoxicity, but CD112 KO as well as double KO significantly reduced cytotoxicity. When targeting KG-1, both CD155 KO and CD112 KO reduced cytotoxicity, with CD112 KO tending to reduce it more, and double KO reducing the cytotoxicity yet further (Figure 6A).

Next, the expression of CD107a, TNFα, and IFNγ was analyzed using flow cytometry when NK-92 DNAM-1 was co-cultured with the leukemia cell lines. The expression was significantly lower when co-cultured with double KO cells, compared with that in wild-type cells. In CD155 or CD112 single KO cells, changes in CD107a and cytokine expression differed between the cell lines (Figure 6B).

These results suggest that the enhancement of cytotoxicity by DNAM-1 induction depends on pathways mediated by CD155 and CD112, and that the dependence on CD155 or CD112 differs between each cell line.

### NK-92 DNAM-1 exerts cytotoxicity against primary acute myeloid leukemia blasts

Using flow cytometry, we next compared the cytotoxicity of NK-92 and NK-92 DNAM-1 against leukemia cells derived from patients with AML.

Cytotoxicity against primary AML blasts with at least 40%

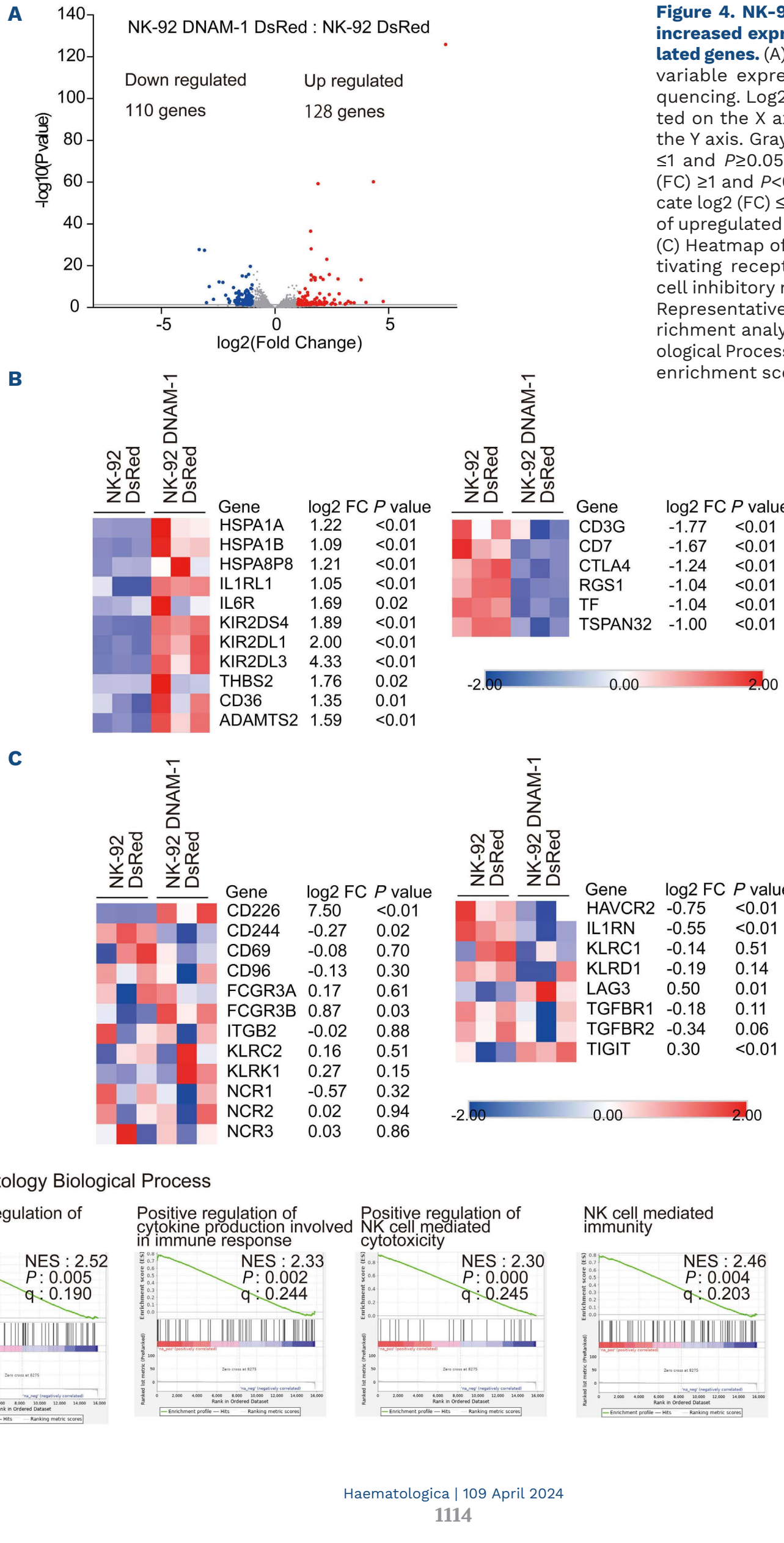


**Figure 4. NK-92 DNAM-1 DsRed showed increased expression of cytotoxicity-related genes.** (A) Volcano plot of genes with variable expression based on RNA-sequencing. Log2 (fold change [FC]) is plotted on the X axis and -log10 (*P* value) on the Y axis. Gray points indicate |log2 (FC)| ≤1 and *P*≥0.05, red points indicate log2 (FC) ≥1 and *P*<0.05, and blue points indicate log2 (FC) ≤ -1 and *P*<0.05. (B) Heatmap of upregulated and downregulated genes. (C) Heatmap of natural killer (NK) cell activating receptor-related genes and NK cell inhibitory receptor-related genes. (D) Representative data from a gene set enrichment analysis of a Gene Ontology Biological Process gene set. NES: normalized enrichment score, q: false discovery rate.

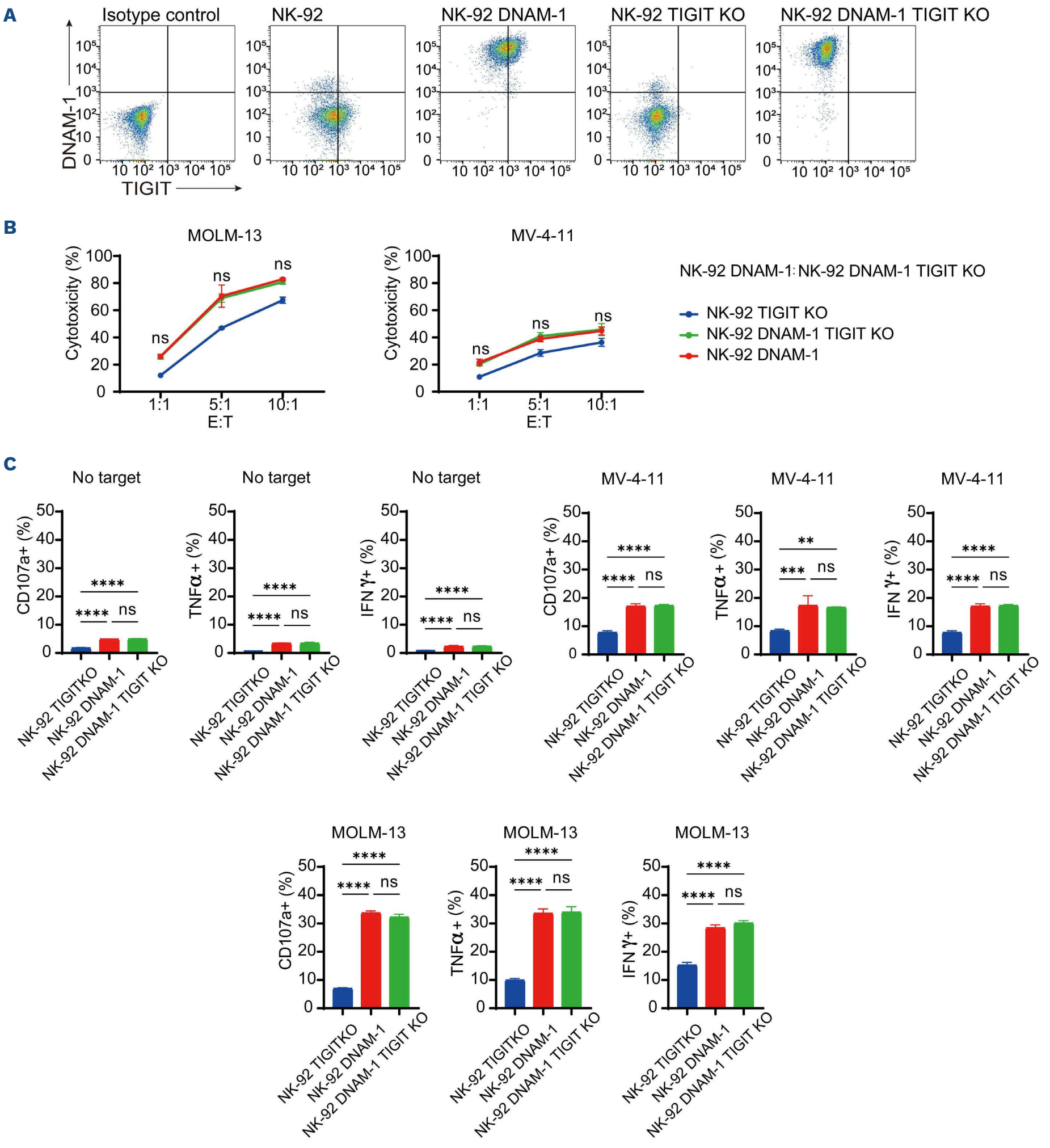


**Figure 5. DNAM-1 transduction and TIGIT knockout do not appear to have an additive effect.** (A) A NK-92 DNAM-1 TIGIT knockout (KO) cell line was generated and its expression was analyzed using flow cytometry. (B) Cytotoxicity of NK-92 DNAM-1, NK-92 TIGIT KO, and NK-92 DNAM-1 TIGIT KO against leukemia cell lines (MOLM-13 and MV-4-11) was analyzed and compared using cytotoxicity assays. Red lines indicate NK-92 DNAM-1, blue lines indicate NK-92 TIGIT KO, and green lines indicate NK-92 DNAM-1 TIGIT KO. (C) CD107a expression of NK-92 DNAM-1, NK-92 TIGIT KO, and NK-92 DNAM-1 TIGIT KO in co-culture with leukemia cell lines was analyzed using flow cytometry. Tumor necrosis factor-α and interferon-γ expression in NK-92 DNAM-1, NK-92 TIGIT KO, and NK-92 DNAM-1 TIGIT KO was analyzed using intracellular flow cytometry. The mean ± standard error are shown. A two-tailed Student *t* test was used to compare two groups, and one-way analysis of variance was used for comparisons among three groups. Multiple comparisons were made using the Tukey test. ns: no statistically significant difference, ***P*<0.01, ****P*<0.001, *****P*<0.0001. TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ.

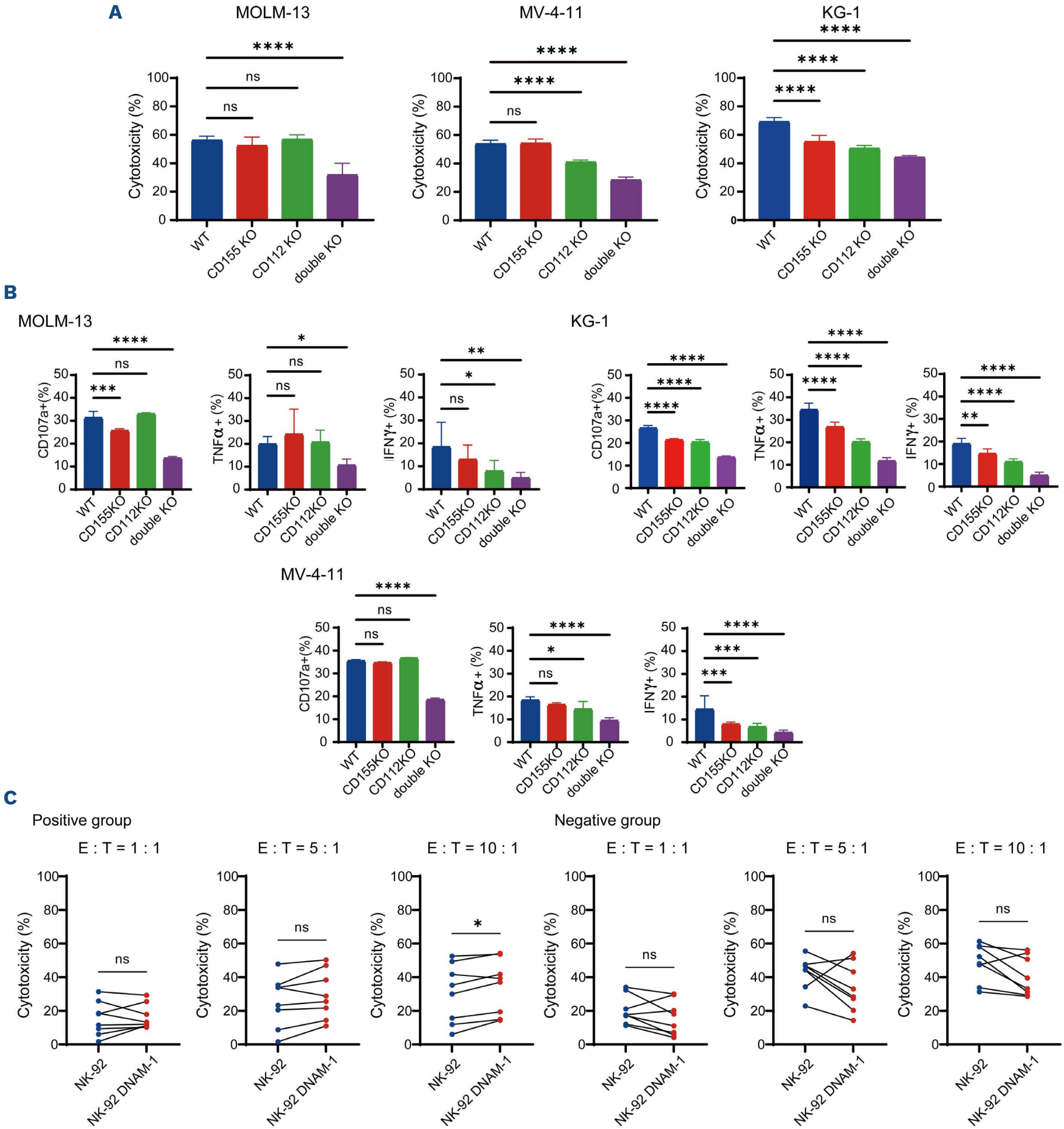


**Figure 6. Enhancement of cytotoxicity by DNAM-1 is dependent on Nectin/Nectin-like family molecules.** (A) Cytotoxicity of NK-92 DNAM-1 against MOLM-13, MV-4-11, KG-1 and CD155 and/or CD112 knockout (KO) cells was analyzed and compared using cytotoxicity assays. (B) CD107a expression of NK-92 DNAM-1 cells co-cultured with MOLM-13, MV-4-11, KG-1 and CD155 and/or CD112 KO cells was analyzed using flow cytometry. Tumor necrosis factor-α and interferon-γ expression of NK-92 DNAM-1 cells was analyzed using intracellular flow cytometry. The mean ± standard error are shown. A two-tailed Student *t* test was used to compare the two groups. (C) Cytotoxicity of NK-92 and NK-92 DNAM-1 against primary acute myeloid leukemia (AML) blasts that expressed at least 40% positivity for CD155 and CD112 and 70% for either (positive group) was compared with that against primary AML blasts with less CD155 and CD112 positivity (negative group). Each point connected by a line represents a sample from the same patient. The mean ± standard error are shown. The Wilcoxon signed-rank test was used for comparison between the two groups. ns: no statistically significant difference, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. WT: wild-type; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; E:T: effector-to-target cell ratio.

and 70% positivity for CD155 and CD112, respectively (positive group), was compared with that against primary AML blasts with less CD155 and CD112 positivity (negative group). In the positive group, the cytotoxicity of NK-92 DNAM-1 was significantly enhanced compared to that of NK-92 at an effector-to-target cell ratio of 10. No significant difference was observed in the negative control group at any effector-to-target cell ratio (Figure 6C).

Thus, the cytotoxicity of NK-92 DNAM-1 was enhanced compared with that of NK-92 in primary AML blasts with strong expression of CD155 and CD112.

### DNAM-1 induced NK-92 displays stronger anti-acute myeloid leukemia effects than NK-92 in a xenograft model

To evaluate the cytotoxicity of NK-92 DNAM-1 *in vivo*, we generated a xenograft mouse model. NSG mice were irradiated on day -1. Luciferase-introduced MOLM-13 ($1\times10^5$ cells/100 μL) were injected via the tail vein with 100 μL of PBS, NK-92 ($5\times10^6$ cells/100 μL), or NK-92 DNAM-1 ($5\times10^6$ cells/100 μL) on day 0. Then, 200 μL of PBS, NK-92 ($5\times10^6$ cells), or NK-92 DNAM-1 ($5\times10^6$ cells) were injected via the tail vein on days 1 and 2. Proliferation of leukemic cells was evaluated using IVIS once or twice a week (Figure 7A).

Representative IVIS images from days 5 and 8 are shown for the three groups: vehicle (PBS), NK-92, and NK-92 DNAM-1. The NK-92 DNAM-1 group showed suppressed AML progression, with this effect being more pronounced than in the PBS or NK-92 groups (Figure 7B).

Regarding average radiance, the NK-92 DNAM-1 group showed significantly suppressed AML progression compared to the vehicle and NK-92 groups on day 15 (Figure 7C). A significant survival benefit was observed (Figure 7D). There was no significant difference in body weight between the three groups (Figure 7E).

## Discussion

This is the first study to analyze the association between the Nectin/Nectin-like family molecules expressed in AML cells and their receptors on NK cells and to demonstrate the utility of treatment with receptor-modified NK cells. The development of immunotherapy for AML has lagged behind that for other hematologic malignancies because of the lack of appropriate therapeutic targets. We, therefore, sought promising targets, which are especially specific to AML patients with a poor prognosis.

NK cells from patients with AML have a suppressive phenotype as shown by the increased expression of TIGIT[36] and decreased expression of DNAM-1,[37] and that prognosis is poor for patients with such impaired immune cells.[36,38]

With regard to CD155 and CD112, there have been reports that leukemia cells escape NK-cell attack by decreasing CD155/CD112 expression,[39] and that the expression of multiple NK-cell receptor ligands, including MICA, ULBP, CD155, and CD112, is associated with prognosis in patients with AML.[40] However, there have been no reports on the association of CD155 or CD112 alone with prognosis in patients with AML or on NK-cell therapy targeting them.

Here, we found that high expression of CD112 is a poor prognostic marker in AML, which is at least partially explained by a higher frequency of cases with high-risk cytogenetics and trends to a higher frequency of *TP53* mutations. In addition, the group with high CD112 expression had fewer *FLT3*-mutated cases and these cases do not benefit from the practically available treatment of FLT3 inhibition. Thus, adaptive immunotherapy that stimulates NK cells through activation of the DNAM-1-CD112 axis by exploiting the high expression of CD112 could be a sophisticated strategy to take advantage of the feature of this group of AML patients with a poor prognosis.

DNAM-1 and TIGIT, the receptors focused on in this study, share some ligands and transmit opposing signals to NK cells.[22] Previous reports indicated that TIGIT inhibits DNAM-1 directly,[41] suggesting that high TIGIT expression may be associated with low DNAM-1 expression, but no such correlation was observed, at least in the samples in this study. By accumulating more cases, it may be possible to analyze whether the expression of DNAM-1 or TIGIT and the correlation between them are related to the remission rate or the subsequent relapse rate.

In the analysis of the antitumor effects of DNAM-1 induction and TIGIT KO, we focused on the DNAM-1 pathway because the effect of DNAM-1 induction was stronger than that of TIGIT KO. Induction of DNAM-1 in NK-92 resulted in increased expression of HSP-related genes, such as *HSPA1A* and *HSPA1B*. HSP contributes to increased immune cell cytotoxicity,[42,43] which may induce the production of pro-inflammatory cytokines such as IL-1 and IL-6, thereby increasing NK-cell activity. In contrast, as negative feedback to the activation by DNAM-1 induction, increased expression of some inhibitory KIR-related genes was observed. Inhibition of these receptors, rather than TIGIT, may be more effective when combined with NK-92 DNAM-1 cell therapy.

No genes and pathways that were downregulated were found to be associated with the suppression of NK-cell function, supporting the usefulness of DNAM-1 induction.

The enhanced cytotoxicity induced by DNAM-1 was dependent on binding to CD155 or CD112. The dependence on CD155 and CD112 was cell line-dependent. In some cell lines, CD112 KO reduced the cytotoxicity of NK-92 DNAM-1, suggesting the importance of the DNAM-1/CD112 axis. It is important to analyze the association between known AML risk factors and CD112 to better select patients who will benefit from treatment with NK-92 DNAM-1. CD112 expression may be a marker for predicting the therapeutic response to DNAM-1-induced NK-cell therapy.

*In vivo*, DNAM-1-induced NK-92 was able to inhibit AML growth compared to the vehicle, but administration of NK-92 DNAM-1 after AML progression did not have any suppressive effect (*data not shown*). In clinical settings, it

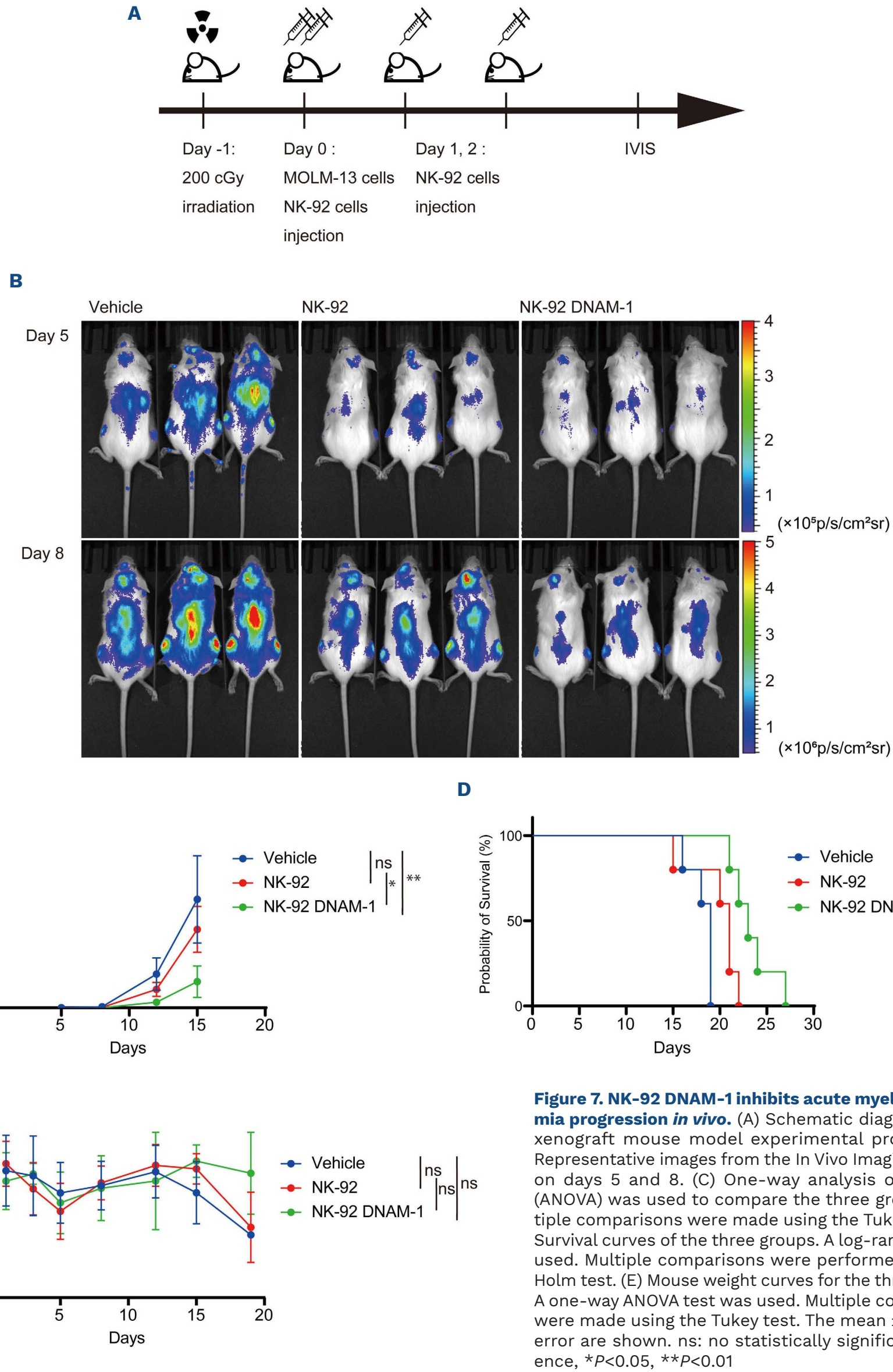


**Figure 7. NK-92 DNAM-1 inhibits acute myeloid leukemia progression *in vivo*.** (A) Schematic diagram of the xenograft mouse model experimental protocol. (B) Representative images from the In Vivo Imaging System on days 5 and 8. (C) One-way analysis of variance (ANOVA) was used to compare the three groups. Multiple comparisons were made using the Tukey test. (D) Survival curves of the three groups. A log-rank test was used. Multiple comparisons were performed with the Holm test. (E) Mouse weight curves for the three groups. A one-way ANOVA test was used. Multiple comparisons were made using the Tukey test. The mean ± standard error are shown. ns: no statistically significant difference, **P*<0.05, ***P*<0.01

is realistic to use NK-92 DNAM-1 cell therapy to eliminate minimal residual disease and for maintenance therapy after remission, but not as induction therapy.

Enhancement of directivity to the tumor is important for enhancing the efficacy of NK-cell therapy. As a method to increase directivity against tumors, the generation of chimeric antigen receptor-NK cells, a combination of antibodies with antibody-dependent cellular cytotoxicity, and a combination of immunostimulatory drugs is encouraged.[44,45] The combination of these drugs may increase the sensitivity of AML cells to NK-92 DNAM-1.[46,47]

In this study, we showed that the Nectin/Nectin-like family molecule CD112 was expressed in AML blasts and that it may be associated with prognosis and therapeutic response. NK-cell therapy that targets these cells may be effective in suppressing AML progression. We propose that the necessary steps before proceeding to a first-in-human study of NK-92 DNAM-1 must include genetic modifications, such as enabling the autocrine secretion of cytokines that activate NK-92 and maintain their proliferation, and introducing suicide genes that prevent tumorigenesis, as well as experiments with patient-derived xenograft models. Moreover, efficacy and safety analyses, including effects on normal hematopoiesis, are needed. Clinically, NK-92 DNAM-1 cell therapy may contribute to the eradication of minimal residual disease as a maintenance therapy after hematopoietic stem cell transplantation and chemotherapy.

**Disclosures**

*YN received research funding from Daiichi Sankyo and an honorarium from Fuji Pharma. YN is a consultant for Novartis and Otsuka Pharmaceutical. KM has received research funding from Nippon Shinyaku, Takeda Pharmaceutical, Kyowa Kirin, Chugai Pharmaceutical, Sumitomo Dainippon Pharma, and Daiichi Sankyo. KM is a consultant for Novartis Pharma, Kyowa Kirin, and Bristol-Myers Squibb. YI received research funding from JSPS KAKENHI (grant number 20K08726), Takeda Pharma, and Bristol Myers Squibb. YI has received honoraria from Bristol Myers Squibb, Takeda Pharma, Janssen Pharmaceutical, and Sanofi. The remaining authors have no conflicts of interest to disclose.*

**Contributions**

*YK and YI performed the research, analyzed and interpreted the data, and wrote the manuscript. YI designed the study and edited the manuscript. ES, SG, YN, and HT analyzed and interpreted the data and revised the manuscript. FN, YT, MS, SY, HY, and KM provided the samples from AML patients for this study. YI supervised the project. YK and YI confirmed the authenticity of all the raw data. All the authors have read and approved the final manuscript.*

**Acknowledgments**

*The authors thank all patients who participated in this study.*

**Funding**

*This study was funded by the Japan Society for the Promotion of Science, Kagaku Kenkyu hi (grant n. 20K08726).*

**Data-sharing statement**

*The data generated in the current study are available from the corresponding author on reasonable request. RNA sequencing data were deposited in the DNA Data Bank of Japan (accession number DRA 015488).*

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