

ORIGINAL ARTICLE

Activated invariant natural killer T cells directly recognize leukemia cells in a CD1d-independent manner

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Abstract

Invariant natural killer T (iNKT) cells are innate-like CD1d-restricted T cells that express the invariant T cell receptor (TCR) composed of V α 24 and V β 11 in humans. iNKT cells specifically recognize glycolipid antigens such as α -galactosylceramide (α GalCer) presented by CD1d. iNKT cells show direct cytotoxicity toward CD1d-positive tumor cells, especially when CD1d presents glycolipid antigens. However, iNKT cell recognition of CD1d-negative tumor cells is unknown, and direct cytotoxicity of iNKT cells toward CD1d-negative tumor cells remains controversial. Here, we demonstrate that activated iNKT cells recognize leukemia cells in a CD1d-independent manner, however still in a TCR-mediated way. iNKT cells degranulated and released Th1 cytokines toward CD1d-negative leukemia cells (K562, HL-60, REH) as well as α GalCer-loaded CD1d-positive Jurkat cells. The CD1d-independent cytotoxicity was enhanced by natural killer cell-activating receptors such as NKG2D, 2B4, DNAM-1, LFA-1 and CD2, but iNKT cells did not depend on these receptors for the recognition of CD1d-negative leukemia cells. In contrast, TCR was essential for CD1d-independent recognition and cytotoxicity. iNKT cells degranulated toward patient-derived leukemia cells independently of CD1d expression. iNKT cells targeted myeloid malignancies more than acute lymphoblastic leukemia. These findings reveal a novel anti-tumor mechanism of iNKT cells in targeting CD1d-negative tumor cells and indicate the potential of iNKT cells for clinical application to treat leukemia independently of CD1d.

KEYWORDS

CD1d, CD2, leukemia, LFA-1, natural killer T-Cells

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1 | INTRODUCTION

Invariant natural killer T (iNKT) cells are an innate-like lymphocyte population defined by their invariant T cell receptors (TCR) composed of V α 24 and V β 11 in humans.¹⁻⁵ iNKT cells recognize glycolipid antigens such as α -galactosylceramide (α GalCer) presented by the MHC class I-like molecule CD1d.¹⁻⁷ Upon activation by TCR-mediated stimulation or inflammatory cytokines, iNKT cells release a large amount of cytokines,¹⁻⁴ which, in turn, activate other cytotoxic immune cells and induce long-term memory CD8 T cell responses.⁸⁻¹⁰ iNKT cells play a role in cancer immunity and exhibit anti-tumor effects through three mechanisms: (i) direct cytotoxicity; (ii) recruitment and activation of other innate and adaptive immune cells by producing Th1 cytokines and maturing dendritic cells; and (iii) regulating immunosuppressive cells in the tumor microenvironment.^{3,4} iNKT cells also function in immunosurveillance against different types of solid and hematological tumors.^{11,12}

Because the invariant V α 24V β 11-TCR recognizes glycolipid antigens presented by CD1d, direct recognition and cytotoxicity of iNKT cells to CD1d-expressing tumor cells are well known.^{6,7,13-16} However, limited numbers of CD1d-expressing leukemia cases are encountered in the clinical setting.¹⁷ While CD1d-independent cytotoxicity in hematopoietic tumor cell lines has been reported from a few laboratories,^{18,19} the cytotoxicity was not reproduced in other laboratories.^{6,7} So far, whether iNKT cells directly recognize CD1d-negative tumor cells has remained unknown.^{3,4} Here, we evaluated the CD1d-independent leukemia recognition of iNKT cells using degranulation and cytokine release assays. iNKT cells have perforin-mediated cytotoxicity against hematopoietic malignancies,^{18,19} and the CD107a degranulation assay reflects ligand recognition and perforin-mediated cytotoxicity in cytotoxic immune cells.^{20,21} We found that iNKT cells recognized leukemia cells in a CD1d-independent manner and that the CD1d-independent recognition depended on V α 24V β 11-TCR.

2 | MATERIALS AND METHODS

2.1 | Cell lines

Leukemia cell lines (K562, chronic myeloid leukemia; HL-60, acute myeloid leukemia; Jurkat, acute T-cell lymphoblastic leukemia) were originally from the ATCC. REH (acute lymphoblastic leukemia) and U937 (histiocytic lymphoma) cell lines were obtained from Cell Line Service and JCRB Cell Bank, respectively. To generate CD1d-transfected K562 cells, pCMV6-XLA4/hCD1d (OriGene Technologies) was transfected into K562 cells with the Neon Transfection System (Invitrogen), and CD1d-expressing K562 cells were sorted by ARIA III (BD Biosciences). All cell lines were cultured in complete medium comprising RPMI 1640 medium (Wako), L-glutamine, penicillin G, streptomycin,

2-mercaptoethanol, HEPES buffer (Invitrogen) and heat-inactivated 10% FCS (Equitech-Bio).

2.2 | Antibodies and flow cytometry

Allophycocyanin (APC)-labeled anti-CD1d antibody (clone 51.1, eBioscience) and IgG2b isotype (clone MPC-11, BioLegend) were used to detect CD1d expression. FITC-labeled anti-V α 24 (clone C15, Beckman Coulter), phycoerythrin (PE)-labeled anti-V β 11 (clone C21, Beckman Coulter) and APC-Cy7-labeled anti-CD3 (clone HIT3a, BioLegend) antibodies, and APC-labeled α GalCer-loaded CD1d-tetramer (Proimmune) were used to identify iNKT cells. The other antibodies used in this study are listed in Table S1. Propidium iodide (PI) solution (Sigma-Aldrich) was used to identify dead cells. Flow cytometric data were acquired with FACSVerse or LSRFortessa-X20 instrument (BD Biosciences) running BD FACSuite or FACSDiva, respectively, and analyzed with Flowjo software (FlowJo, LLC).

2.3 | Cell preparation

Purified iNKT cells were generated as previously described.²² Briefly, venous blood was obtained from healthy adult volunteer donors after obtaining written informed consent, and PBMC were separated by density gradient centrifugation using Ficoll-Paque (GE Healthcare). PBMC were cultured in complete RPMI 1640 medium for 9-14 days in the presence of 100 U/mL of recombinant human IL-2 (Shionogi) and 200 ng/mL of α GalCer (REGIMMUNE, Tokyo, Japan). The iNKT cells were then isolated with an autoMACS Pro Separator (Miltenyi Biotec) using FITC-labeled anti-V α 24 antibody and anti-FITC microbeads (Miltenyi Biotec). The purified iNKT cells were cultured in complete medium containing IL-2 until used in experiments (within 4 days after isolation). We obtained patient-derived leukemia cells from pediatric patients with untreated leukemia after obtaining written informed consent. All studies were approved by the institutional review board of Chiba University.

2.4 | In vitro cytotoxicity assay

Target cells were labeled with CellTrace Violet (CTV, Invitrogen) for identification according to the manufacturer's protocol. CTV-labeled target cells (4×10^4) and purified iNKT cells were co-incubated for 4 hours at 37°C in complete medium at the indicated effector to target cell (ET) ratio. After the incubation, FITC-labeled Annexin V (BioLegend) and PI in Annexin V Binding Buffer (BioLegend) were added to identify apoptotic cells according to the manufacturer's protocol. Annexin V/PI⁺ cells were counted as living cells. Cytotoxicity (%) was calculated as $100 \times (1 - (\text{living cells without effector cells} - \text{living cells with effector cells}) / \text{living cells without effector cells})$.

2.5 | Degranulation assay

Purified iNKT cells ($1-2 \times 10^5$) were incubated for 2 hours at 37°C in complete medium in the presence of 2.5 µg anti-CD107a antibody (clone H4A3, BioLegend) and 2 µmol/L monensin (BioLegend) with 4×10^4 target cells or with cross-linking natural killer (NK) cell-activating receptors. After incubation, iNKT cells were collected and stained with anti-Vα24 and anti-CD3 antibodies for 20 minutes. CD107a expression indicates the percentage of CD107a positive fraction induced by adding target cells or cross-linking receptors compared with unstimulated iNKT cells. The gating strategy is shown in Figure S1.

2.6 | Cytokine measurement

The indicated numbers of purified iNKT cells were incubated for 4 or 24 hours at 37°C in 200 µL complete medium with 4×10^4 target cells or with cross-linking NK cell-activating receptors. After incubation, the supernatant was collected, and interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) were measured by cytometric bead array according to the manufacturer's protocol. Data were acquired on FACSVerse and analyzed with FCAP Array software (BD Biosciences).

2.7 | Blocking assay and cross-linking receptors

For blocking receptors, iNKT cells were pre-incubated for 30 minutes with 10 µg/mL blocking antibodies at 37°C. For cross-linking receptors, iNKT cells were pre-incubated for 30 minutes with 10 µg/mL primary antibodies at 37°C followed by 19 µg/mL secondary goat F(ab')₂ anti-mouse IgG (Jackson Laboratories). As a control, the IgG1 antibody was used. The antibodies used for blocking and cross-linking receptors are listed in Table S1.

2.8 | Mice

NOG mice were purchased from In-Vivo Science. Mice were maintained under specific pathogen-free conditions and studied in compliance with institutional guidelines for the care and use of laboratory animals. All animal procedures were approved by the Chiba University Institutional Animal Care and Use Committee.

2.9 | In vivo cytotoxicity assay

Six-week-old to eight-week-old female NOG mice were intravenously inoculated with 1×10^6 K562 cells resuspended in 200 µL PBS per mouse. On the following day, each mouse was intravenously injected with 4×10^6 purified iNKT cells (in the treatment group) or PBS (in the control group). Bodyweight was monitored twice a week. Mice were humanely euthanized if they lost more than 20% of their peak weight.

2.10 | Quantitative real-time PCR

Quantitative RT-PCR was performed as previously described.^{22,23} The primers were designed using the Universal ProbeLibrary System Assay Design (https://lifescience.roche.com/global_en/articles/Universal-ProbeLibrary-System-Assay-Design.html) and ordered from Sigma-Aldrich. The primer sequences and probe used were as follows: *CD1D*, 5'-TGAGTTGCCAAAGAGGAGTCT-3', 5'-GCCGCTCACTCAGTAGGTT-3', and universal probe #65 (Roche, Basel, Switzerland). *GAPDH* housekeeping gene (TaqMan Pre-Developed Assay Reagent, Applied Biosystems, Foster City) was used as an internal control. The following thermal profile was used: initial denaturation at 95°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 1 second and annealing at 60°C for 25 seconds.

2.11 | CRISPR/Cas9-mediated genome editing

CRISPR RNA (crRNA) were designed using the online tool provided by CHOPCHOP (<http://chopchop.cbu.uib.no>) and purchased from Integrated DNA Technologies. Negative control crRNA #1 and *trans*-activating crRNA (tracrRNA) were obtained from Integrated DNA Technologies. The crRNA:tracrRNA complex was generated by incubation at 95°C for 5 minutes according to the manufacturer's protocol. Cas9/gRNA ribonucleoproteins (RNP) were prepared immediately before the experiments by incubating 1250 ng Cas9 protein (TrueCut v2, Invitrogen) with 250 ng crRNA:tracrRNA complex in 5 µL transfection buffer at room temperature for 10 minutes. Purified iNKT cells or cell lines were electroporated with RNP and Alt-R Cas9 Electroporation Enhancer (Integrated DNA Technologies) using the Neon Transfection System (Invitrogen). In cell lines, KO-cells were established from a single cell. TCR-KO iNKT cells were generated by knocking out TCR alpha or beta constant (TRAC or TRBC). Purified iNKT cells were electroporated on the day of magnetic cell sorting and used in experiments 3 or 4 days later. The sequences of the crRNA are as follows: *CD1D*, 5'-CGTCCTTCGCCAATAGCAGC-3' and 5'-CTGGTCGCTGAACGTGCCCT-3'; *B2M*, 5'-ACTCACGC TGGATAGCCTCC-3' and 5'-GAGTAGCGCGAGCACAGCTA-3'; *CD2*, 5'-ACGAATGCCTTGAAACCTG-3'; *CD11A* (*ITGAL*), 5'-CGTTTCCGACCTGCAGGACG-3'; *TRAC*, 5'-TGTGCTAGAC ATGAGGTCTA-3'; and *TRBC*, 5'-GCAGTATCTGGAGTCATTGA-3'.

2.12 | Live cell imaging

Target cells were labeled with Calcein-AM (Abcam, Cambridge) for identification according to the manufacturer's protocol. iNKT cells (1×10^5) and labeled target cells (4×10^4) were mixed and co-cultured for 30 minutes in a glass-bottom 96-well plate on BZ-X800 (Keyence, Osaka, Japan) equipped with a 5% CO₂ incubation chamber before image acquisition. Time-lapse imaging

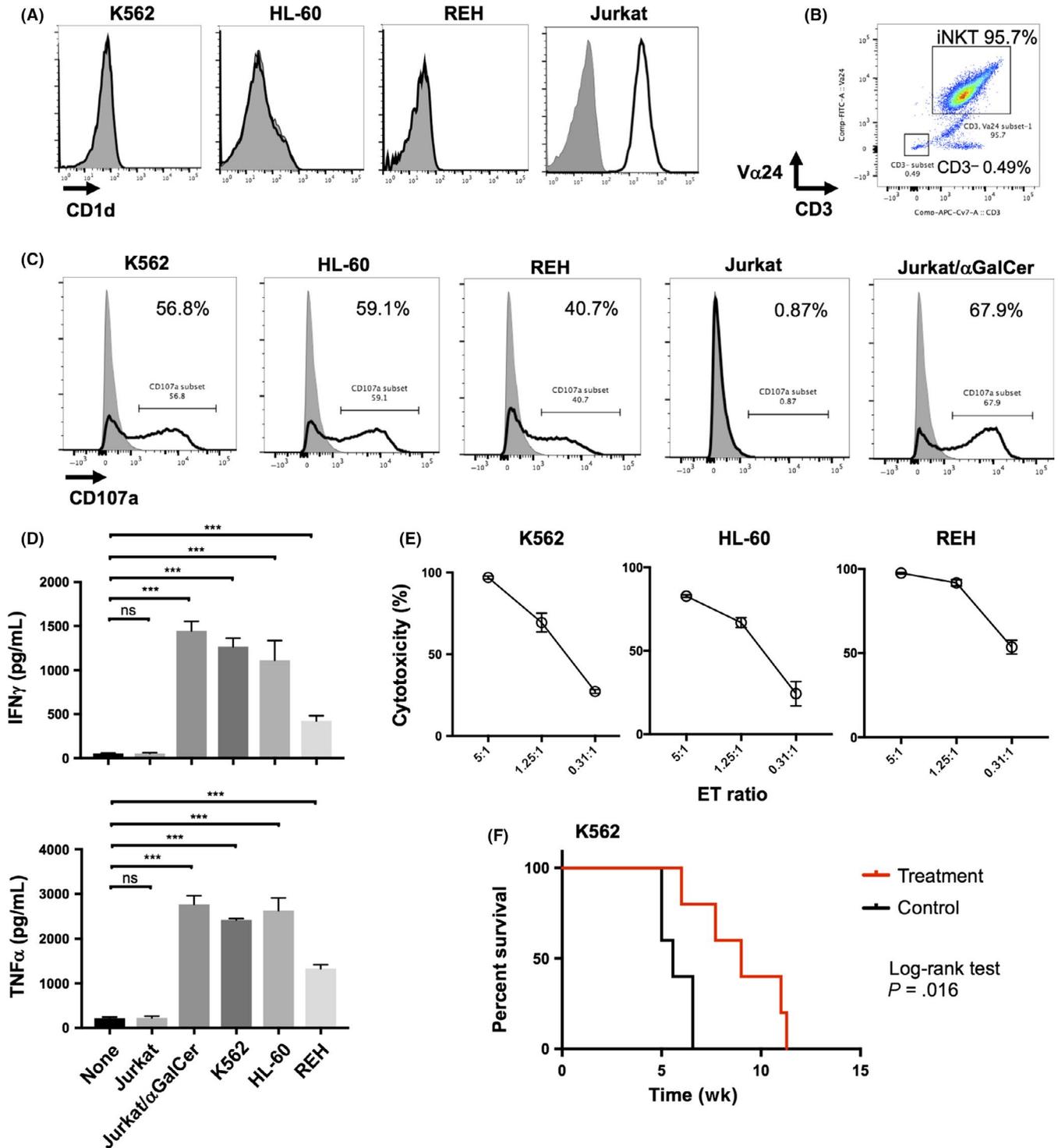


FIGURE 1 Invariant natural killer T (iNKT) cells recognize CD1d-negative leukemia cells and show direct cytotoxicity. A, Flow cytometry analysis of surface CD1d expression on leukemia cell lines (K562, HL-60, REH and Jurkat). Isotype, regular line indicated by gray filled region; CD1d, bold line. B, Representative data of purified iNKT cells after magnetic-activating cell sorting (lymphocyte/PI⁺). C, Representative flow cytometry analysis of degranulation assay of purified iNKT cells (lymphocyte/PI⁺/CD3⁺/Vα24⁺/singlet cells, Figure S1). iNKT cells alone, regular line indicated by gray filled region; iNKT cells co-cultured with leukemia cells, bold line. Numbers indicate the percent of iNKT cells with CD107a expression induced by leukemia cells. D, Production of Th1 cytokines after 2 × 10⁵ iNKT cells were co-cultured with leukemia cell lines for 24 h. Data are shown as mean ± SD from three technical replicates and are representative of two biologically independent experiments. Two-tailed unpaired Student's *t* test was used (****P* < 0.001). ns, not significant. E, In vitro cytotoxicity of iNKT cells on the indicated cell lines after 4 h. Data are shown as mean ± SD from three technical replicates and are representative of two biologically independent experiments. F, In vivo cytotoxicity of iNKT cells. Survival curves of K562 cell-inoculated NOG mice that received iNKT cell transfer treatment (red line) or PBS as control (black line) (*n* = 5 mice/group). ET ratio, effector to target cell ratio; IFN γ , interferon- γ ; TNF α , tumor necrosis factor- α

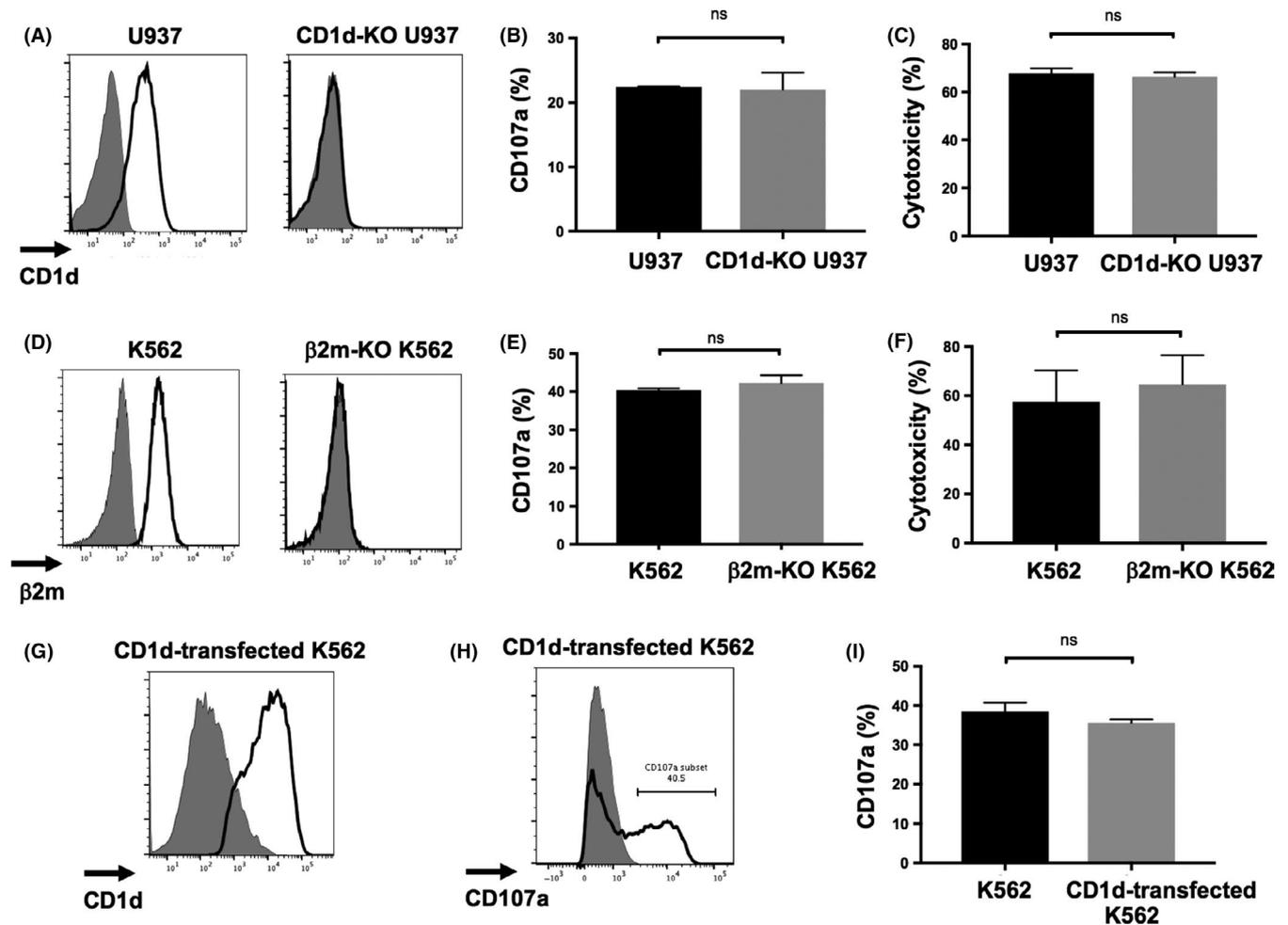


FIGURE 2 Confirmation of direct CD1d-independent cytotoxicity and recognition of invariant natural killer T (iNKT) cells. A, Flow cytometry analysis of surface CD1d expression on wild-type and CRISPR/Cas9-mediated CD1d-KO U937 cells. Isotype, regular line indicated by gray filled region; CD1d, bold line. B, Degranulation assay of iNKT cells toward wild-type U937 and CD1d-KO U937 cells. C, Cytotoxicity of iNKT cells toward wild-type U937 and CD1d-KO U937 cells. ET ratio was 5:1. D, Flow cytometry analysis of surface beta2m expression on wild-type and CRISPR/Cas9-mediated beta2m-KO K562 cells. Isotype, regular line indicated by gray filled region; beta2m, bold line. E, Degranulation assay of iNKT cells toward wild-type K562 and beta2m-KO K562 cells. F, Cytotoxicity of iNKT cells toward wild-type K562 and beta2m-KO K562 cells. ET ratio was 2.5:1. G, Flow cytometry analysis of surface CD1d expression on CD1d-transfected K562 cells after selection by CD1d expression. Isotype, regular line indicated by gray filled region; CD1d, bold line. H, Representative flow cytometry analysis of degranulation assay with CD1d-transfected K562 cells. I, Degranulation assay of iNKT cells toward wild-type and CD1d-transfected K562 cells. Data are shown as mean \pm SD from three technical replicates are representative of two independent experiments. Two-tailed unpaired Student's *t* test was used in (B, C, E, F and I). ET ratio, effector to target cell ratio; IFN γ , interferon- γ

was captured every 2 minutes for 4 hours (original magnification: $\times 20$). Fluorescence and phase contrast overlay videos were obtained using BZ-H4XT multidimensional time-lapse software (Keyence). Absolute green luminance was obtained using BZ-H4XT software.

2.13 | Statistics

Statistical analyses were performed using GraphPad PRISM7 software (GraphPad) or Microsoft Excel (Microsoft). An unpaired two-sided Student's *t* test or paired *t* test was used to compare data from different experimental conditions.

3 | RESULTS

3.1 | Invariant natural killer T cells recognize CD1d-negative leukemia cells and show direct cytotoxicity

Human leukemia cell lines K562, HL-60 and REH cells did not express CD1d, while Jurkat cells expressed CD1d (Figure 1A). Quantitative RT-PCR did not detect CD1d mRNA in any cell line except for Jurkat cells (data not shown). These data were in line with the Human Protein Atlas (<https://www.proteinatlas.org>). In our cell preparation method, the purity of iNKT cells was approximately 95% (Figure 1B), and CD3 $^+$ /V α 24 $^+$ cells were all V β 11 $^+$ and CD1d-tetramer $^+$ cells (data not shown). To confirm

whether iNKT cells directly recognize CD1d-negative leukemia cells and show direct cytotoxicity, we performed degranulation and cytokine assays. As Jurkat cells with loaded α GalCer induced iNKT cell degranulation, CD1d-negative leukemia cell lines (K562, HL-60 and REH cells) also induced degranulation without α GalCer loading (Figure 1C). Cytokine assay demonstrated that CD1d-negative leukemia cell lines induced Th1 cytokine release from iNKT cells similar to Jurkat cells with α GalCer (Figure 1D). The leukemia cell lines alone did not produce Th1 cytokines in the detectable range (data not shown). The direct cytotoxicity toward CD1d-negative K562 cells was shown by in vitro experiments (Figure 1E) and an in vivo experiment using NOG mice inoculated with K562 cells in veins (Figure 1F). These data indicate

that iNKT cells recognize CD1d-negative leukemia cells. iNKT cell recognition and cytotoxicity toward K562 cells is shown in Video S1 and Figure S2A.

To confirm the CD1d-independent cytotoxicity and recognition of iNKT cells, we established CD1d-KO U937 cells using the CRISPR/Cas9 system (Figure 2A). In contrast to Jurkat cells, CD1d-positive U937 cells induce iNKT cell degranulation without loaded α GalCer. iNKT cells degranulated CD1d-KO U937 cells similar to wild-type U937 cells (Figure 2B). The cytotoxicity of iNKT cells was not different between wild-type U937 and CD1d-KO U937 cells (Figure 2C). We obtained the same results with β 2M-KO K562 cells (Figure 2D–F). Next, to assess whether CD1d expression influences NKT cell degranulation, we

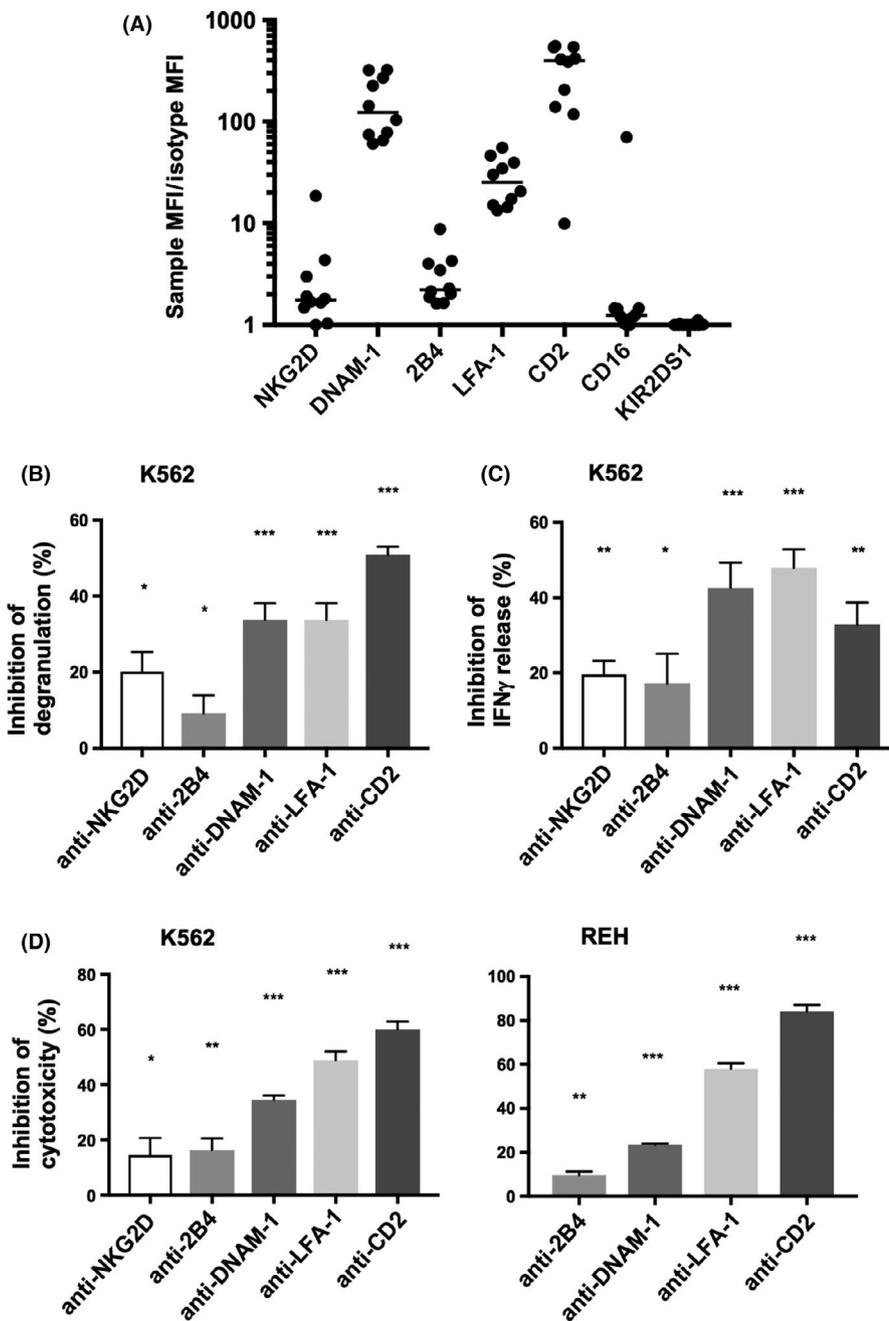


FIGURE 3 Natural killer (NK) cell-activating receptors contribute to invariant natural killer T (iNKT) cell CD1d-independent cytotoxicity. A, Cell surface expression of NK cell-activating receptors on iNKT cells from healthy volunteer donors ($n = 10$). Lines indicate the median relative mean fluorescence intensity (MFI). B, Inhibition rates of induced-CD107a expression in iNKT cells treated with antibodies of NK cell-activating receptors. IgG1 antibody was used as control and inhibition was set as 0%. C, Inhibition rates of release of IFN γ in iNKT cells treated with antibodies of NK cell-activating receptors. IgG1 antibody was used as control and inhibition was set as 0%. D, Inhibition rates of cytotoxicity of iNKT cells treated with antibodies of NK cell-activating receptors toward K562 and REH cells. IgG1 antibody was used as control and inhibition was set as 0%. Data are shown as the mean \pm SD from three technical replicates and representative of two biologically independent experiments. Two-tailed unpaired Student's t test was used (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) in (B–D). IFN γ , interferon- γ

performed a CD107a assay with CD1d-transfected K562 cells. The results showed that CD1d-transfected K562 cells also induced CD107a expression (Figure 2G–I). Together, these results indicate that iNKT cells recognize leukemia cells in a CD1d-independent manner. Furthermore, this CD1d-independent recognition is also independent of all classical and nonclassical MHC class I molecules, such as other CD1 family members and MR1.

3.2 | Natural killer cell-activating receptors contribute to invariant natural killer T cell CD1d-independent cytotoxicity as co-stimulatory receptors

To identify the molecule that contributes to the CD1d-independent recognition, we focused on NK cell-activating receptors.^{23, 24} We first analyzed the expression of NK cell-activating receptors on iNKT cells and found that DNAM1, 2B4, LFA-1 and CD2 were expressed in all donors (Figure 3A). NKG2D expression varied among donors. We next blocked receptors using antibodies and found that degranulation, IFN γ release and direct cytotoxicity of iNKT cells were inhibited upon blocking each of the assessed receptors (Figure 3B–D).

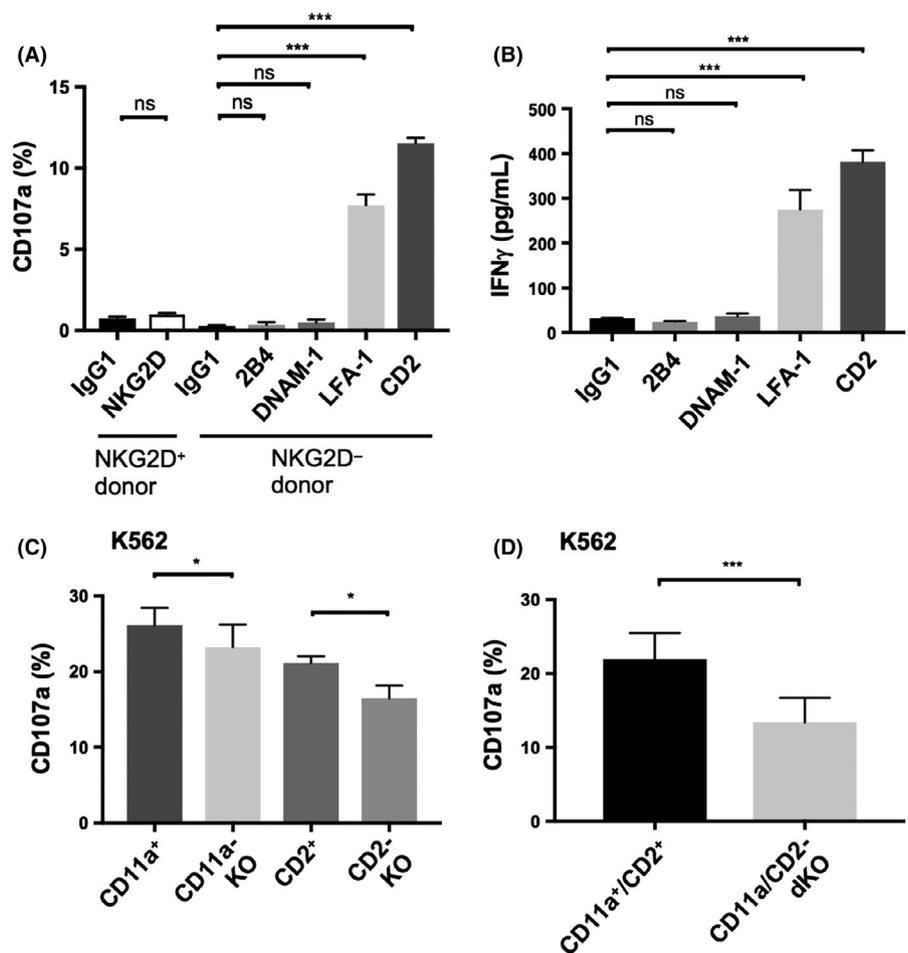
To stimulate iNKT cells with a single receptor, we cross-linked the receptors and found that cross-linking LFA-1 or CD2 induced

degranulation and IFN γ release (Figure 4A, B). To determine whether iNKT cells depend on LFA-1 or CD2 for CD1d-independent recognition, we next knocked out CD11a (LFA-1 alpha chain) and CD2 in iNKT cells. Although CD11a- or CD2-KO iNKT cells showed a reduction in induced-CD107a expression, both iNKT cells still reacted to K562 cells (Figure 4C). CD11a and CD2 double-KO iNKT cells also reacted to K562 cells (Figure 4D). Together, these results indicate that these NK cell-activating receptors contribute to CD1d-independent cytotoxicity and mainly serve as co-stimulatory receptors.

3.3 | T cell receptor is essential for CD1d-independent recognition and cytotoxicity

Next, to investigate whether V α 24V β 11-TCR is responsible for CD1d-independent recognition, we generated TCR-KO iNKT cells using the CRISPR/Cas9 system.²⁵ When we knocked out TCR alpha or beta constant (TRAC or TRBC), both TCR alpha and beta chain and surface CD3 ϵ disappeared on the cell surface (Figure S3). We identified TCR-KO iNKT cells by lymphocyte gating, PI-negative and V α 24-negative cells. TCR-KO iNKT cells were α GalCer-loaded CD1d-tetramer-negative (Figure 5A). We found that TCR-KO iNKT cells did not degranulate toward CD1d-negative leukemia cells

FIGURE 4 Natural killer (NK) cell-activating receptors serve as co-stimulatory receptors in invariant natural killer T (iNKT) cells. A, Degranulation assay of iNKT cells with cross-linking of the indicated receptor. B, Release of IFN γ from 2×10^5 iNKT cells with cross-linking of the indicated receptor for 4 h. C, Degranulation assay using CD11a-KO and CD2-KO iNKT cells toward K562 cells. Transfected CD11a⁺ and CD2⁺ iNKT cells without gene knockout were cultured together and used as control. D, Degranulation assay using CD11a-/CD2-double KO (dKO) iNKT cells toward K562 cells. Transfected CD11a⁺/CD2⁺ iNKT cells without gene knockout were cultured together and used as controls. Data are shown as the mean \pm SD from three technical replicates and are representative of two biologically independent experiments (A–D). Two-tailed unpaired Student's *t* test (A, B) and two-tailed paired *t* test (C, D) were used (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). IFN γ , interferon- γ



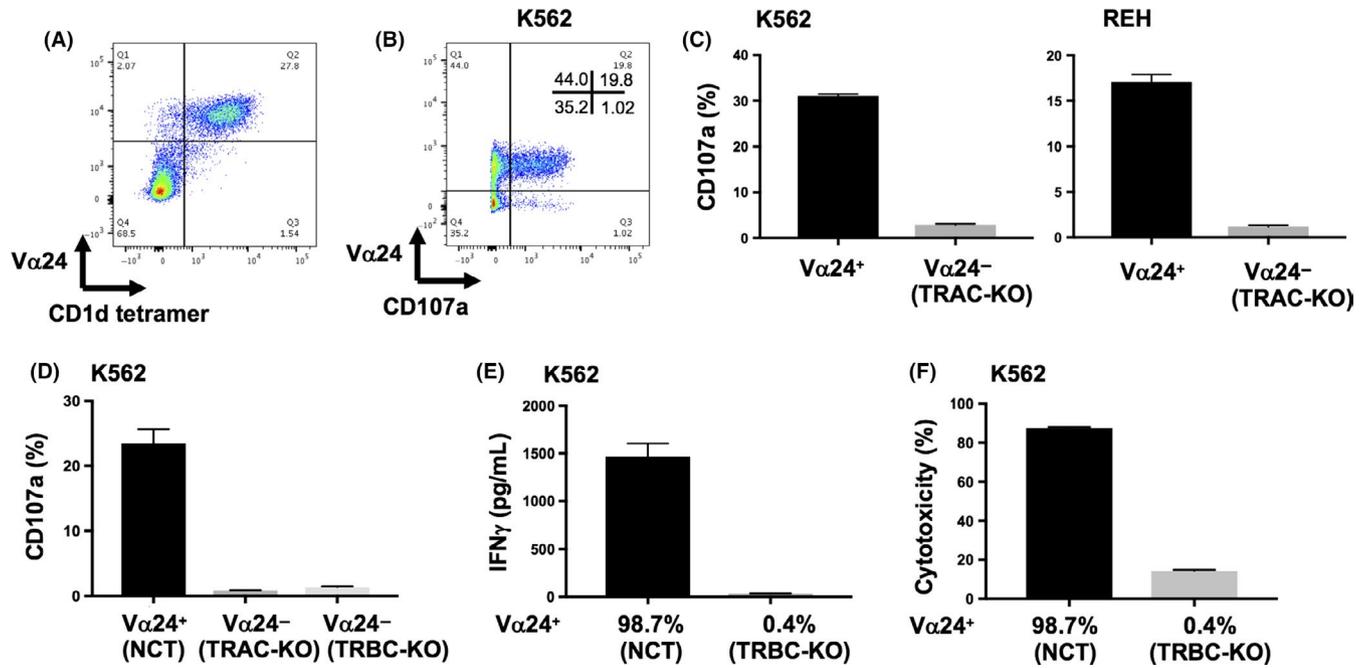


FIGURE 5 T cell receptor (TCR) is essential for CD1d-independent recognition and cytotoxicity. A, Flow cytometry analysis of α GalCer-loaded CD1d-tetramer staining on TCR-KO invariant natural killer T (iNKT) cells (lymphocyte/PI⁺/singlet). B, Representative flow cytometric analysis of degranulation assay using TRAC-KO iNKT cells (CTV/lymphocyte/PI⁺/singlet). Tumor cells were excluded by pre-staining with CellTrace Violet (CTV). C, Summary of degranulation assay of TRAC-KO iNKT cells toward K562 and REH cells. D, Degranulation assay of TRAC- and TRBC-KO iNKT cells. As a control, V α 24⁺ iNKT cells transfected with negative control crRNA (NCT) were used. E, Release of IFN γ from TRBC-KO iNKT after 4-h co-culture with K562 cells. iNKT cells were resorted 3 days after transfection with CRISPR/Cas9 RNP; the V α 24⁺ cell percentages are shown at the bottom. NCT-transfected V α 24⁺ iNKT cells were used as control. F, Cytotoxicity of TRBC-KO iNKT cells toward K562 cells. iNKT cells were prepared as in the previous experiment. NCT-transfected V α 24⁺ iNKT cells were used as control. Data are shown as the mean \pm SD from three technical replicates and are representative of two biologically independent experiments in (C–F)

(Figure 5B, C). The results did not change even with knocking out TRBC (Figure 5D). TCR-KO iNKT cells did not release IFN γ toward K562 cells (Figure 5E). TCR-KO iNKT cells showed decreased cytotoxicity with K562 cells (Figure 5F). Together, these results indicate that iNKT cells depend on the invariant V α 24V β 11-TCR to recognize CD1d-negative leukemia cells, and the CD1d-independent cytotoxicity largely depends on V α 24V β 11-TCR.

3.4 | Invariant natural killer T cells target patient-derived leukemia cells in a CD1d-independent manner

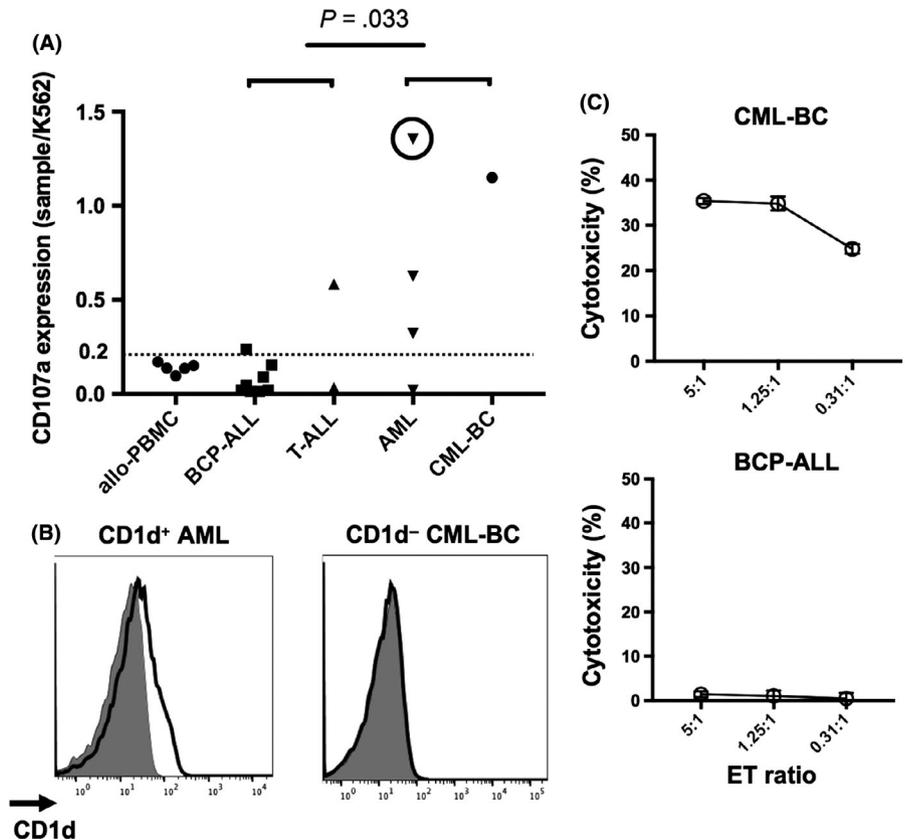
We examined whether iNKT cells target CD1d-negative leukemia cells in patient-derived leukemia cells. We collected PBMC or mononuclear bone marrow cells (MBMC) from 15 pediatric patients including 8 with B-cell precursor acute lymphoblastic leukemia (BCP-ALL), 2 with T-cell ALL (T-ALL), 4 with acute myeloid leukemia (AML) and 1 with chronic myeloid leukemia-blastic crisis (CML-BC); PBMC were obtained from 5 adult healthy donors. Among the patient-derived leukemia samples, 1 was CD1d-positive (Figure 6A). CD1d expression in patient-derived leukemia cells was evaluated with PI⁺/CD45^{dim} cells (Figure 6B, Figure S4). While iNKT cells were less degranulated toward allogeneic (allo)-PBMC from healthy donors, iNKT cells degranulated toward some patient samples

including CD1d-negative leukemia cells (Figure 6A). We adjusted CD107a expression by K562 cells and considered an index >0.2 as positive; two of ten CD1d-negative ALL samples and three of four CD1d-negative myeloid leukemia samples were considered positive. iNKT cells did not aggregate around allogeneic (allo)-PBMC and were not cytotoxic (Video S2 and Figure S2B). Of note, iNKT cells degranulated CD1d-negative myeloid leukemia cells (AML and CML-BC) more than CD1d-negative ALL cells (BCP-ALL and T-ALL) ($P = 0.033$). We confirmed CD1d-independent cytotoxicity toward patient PBMC with CML-BC (Figure 6C, top). iNKT cells were not cytotoxic toward MBMC with BCP-ALL which did not induce degranulation (Figure 6C, bottom).

4 | DISCUSSION

In this study, we provided clear evidence for the ability of iNKT cells to recognize leukemia cells in a cd1d-independent manner. So far, whether iNKT cells recognize CD1d-negative tumor cells has been unknown.^{3,4} Here, we showed that iNKT cells were directly activated by CD1d-negative leukemia cells similar to α GalCer-loaded CD1d-positive leukemia cells using degranulation and cytokine assays. Degranulation and cytokine assay demonstrated that iNKT cells recognized CD1d-negative leukemia cells similar to α GalCer-loaded

FIGURE 6 Invariant natural killer T (iNKT) cells target patient-derived leukemia cells in a CD1d-independent manner. A, Degranulation assay with patient PBMC or MBMC that include leukemia cells without loading α GalCer. ET ratio was 1:1. Circle indicates CD1d-positive leukemia sample. Two-tailed unpaired Student's *t* test was used to compare myeloid leukemia cells (AML and CML-BC) and ALL cells (BCP-ALL and T-ALL). B, Representative flow cytometry analysis of CD1d expression on leukemia cells. Isotype, regular line indicated by gray filled region; CD1d, bold line. Leukemia cells were identified as CD45^{dim}/PI⁻ cells (Figure S4). C, Cytotoxicity toward patient-derived CD1d-negative leukemia cells. PBMC from a patient with CML-BC (top). MBMC from patients with BCP-ALL that did not induce CD107a expression on iNKT cells (bottom). Leukemia cells were identified as CTV⁺/CD34⁺ cells. Data are shown as the mean \pm SD from three technical replicates



CD1d-positive cells. The amounts of released Th1 cytokines toward CD1d-negative leukemia cells were comparable to α GalCer-loaded CD1d-positive cells. iNKT cells show indirect cytotoxicity with adjuvant effects, an ability to induce the activation of other cytotoxic immune cells such as NK cells and cytotoxic T lymphocytes through producing IFN γ .²⁶ Considering that the abundant cytokine production is a crucial characteristic of iNKT cells,²⁷ direct recognition is vital for the anti-tumor role of iNKT cells. Direct cytotoxicity was shown in vivo in NOG mice inoculated with K562 cells. However, this result may underestimate the efficacy of iNKT cell treatment because we cannot evaluate the indirect cytotoxicity in immunodeficient mice and human iNKT cells cannot persist in mice without human cytokines.²⁸

We revealed a role of NK cell-activating receptors expressed on iNKT cells as co-stimulatory receptors. The role of these receptors in iNKT cell cytolytic function has been poorly characterized except for NKG2D.²⁹ We showed that other NK cell-activating receptors such as DNAM-1, 2B4, LFA-1, and CD2 serve as co-stimulatory molecules. Cross-linking experiments showed the potential to recognize tumor cells with LFA-1 and CD2. Cytokine release following cross-linkage of LFA-1 is in line with a recent report demonstrating that high densities of ICAM-1, a ligand of LFA-1, were sufficient to activate iNKT cell cytokine secretion.³⁰ However, TCR-KO iNKT cells did not release IFN γ to ICAM-1-positive K562 cells with LFA-1 in our experiments. Engagement of LFA-1 and ICAM-1 on K562 cells alone may be insufficient to induce cytokine secretion in iNKT cells.

We also found that iNKT cells react to CD1d-negative leukemia cells in a TCR-dependent way. This CD1d-independent recognition was also β 2M-independent, which means that iNKT cells exhibit a mechanism to recognize leukemia cells independently of not only CD1d but also other MHC class I-like antigen presenting molecules. This finding is surprising because glycolipid recognition of iNKT cells was considered to be CD1d-restricted. However, other innate-like T cells of V γ 9V δ 2 cells recognize not only microbes but also leukemia cells in an MHC class I- and CD1-independent manner, but via TCR,^{31,32} and TCR recognizes phosphoantigens bound with BTN3A1.³³ Innate immune cells rely on pattern recognition receptors and elicit prompt responses against cancer-associated danger signals.³⁴ Innate-like T cell subsets may also have pattern recognition-like receptors. Other innate-like T cells of mucin-specific CD8 T cells also showed cytolytic activity independently of HLA class I and CD1.³⁵

Finally, we confirmed iNKT cell CD1d-independent cytotoxicity in patient-derived leukemia cells. The limited number of CD1d-expressing leukemia cases was in line with the previous report.¹⁷ iNKT cells were less cytotoxic toward healthy allo-PBMC but showed cytotoxicity toward patient PBMC, which include leukemia cells, especially myeloid leukemia cells. This finding may indicate that similar to NKG2D³⁶ and V γ 9V δ 2-TCR,³¹ V α 24V β 11-TCR also senses some molecular stress signatures that are absent from healthy cells but are upregulated in malignant or infected cells. This hypothesis supports the findings that iNKT cells have a critical role in tumor immunosurveillance^{11,12,37-39} and that a low number of iNKT cells

is associated with poor survival in AML.^{40,41} In addition, iNKT cells are not HLA-restricted and did not cause or reduce graft versus host disease.^{12,42,43} From these observations, adoptive transfer of allo-iNKT cells that present CD1d-independent cytotoxicity may be an efficient therapy. Furthermore, iNKT cells can be used as an ideal vector for chimeric antigen receptor-T or recombinant TCR-T cell therapies.⁴⁴

We do not yet know the target molecule that iNKT cells recognize independently of CD1d. Still, the recognition differences among healthy PBMC, ALL, and myeloid malignancies may help to identify the target. We found that degranulated iNKT cells were more frequently observed on myeloid malignancies than on healthy PBMC and ALL; we therefore hypothesize that the target is one of molecules that are highly expressed on myeloid malignancies. Another limitation is that we could not assess CD1d-independent recognition in primary iNKT cells because of the small number of iNKT cells in human PBMC from healthy donors. This recognition system may be limited to activated iNKT cells. However, from the viewpoint of the therapeutic use of iNKT cells, the activation and proliferation of iNKT cells are mandatory. It is more important to establish an efficient culture method of iNKT cells maintaining CD1d-independent cytotoxicity in the future.

In conclusion, here we clarified that activated iNKT cells can recognize leukemia cells in a CD1d-independent manner and show direct cytotoxicity toward CD1d-negative leukemia cells. Using the CRISPR/Cas9 system to knock out TCR or NK cell-activating receptors on iNKT cells, we found that $V\alpha 24V\beta 11$ -TCR is responsible for the CD1d-independent leukemia recognition in iNKT cells. These findings established a novel anti-tumor mechanism of iNKT cells and may be the basis for further research regarding a new tumor-associated target recognized by iNKT cells.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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REFERENCES

- Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol*. 2004;4:231-237.
- Zhang Y, Springfield R, Chen S, et al. α -GalCer and iNKT cell-based cancer immunotherapy: Realizing the therapeutic potentials. *Front Immunol*. 2019;10:1126.
- Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol*. 2012;12:239-252.
- Nair S, Dhodapkar MV. Natural killer T cells in cancer immunotherapy. *Front Immunol*. 2017;8:1178.
- Lampy N, Nissen MD, Mattarollo SR. Invariant natural killer T cells in immune regulation of blood cancers: Harnessing their potential in immunotherapies. *Front Immunol*. 2017;8:1355.
- Metelitsa LS, Naidenko OV, Kant A, et al. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol*. 2001;167:3114-3122.
- Haraguchi K, Takahashi T, Nakahara F, et al. CD1d expression level in tumor cells is an important determinant for anti-tumor immunity by natural killer T cells. *Leuk Lymphoma*. 2006;47:2218-2223.
- Fujii S, Shimizu K, Okamoto Y, et al. NKT cells as an ideal anti-tumor immunotherapeutic. *Front Immunol*. 2013;4:409.
- Gottschalk C, Mettke E, Kurts C. The role of invariant natural killer T cells in dendritic cell licensing, cross-priming, and memory CD8⁺ T cell generation. *Front Immunol*. 2015;6:379.
- Dashtsoodol N, Shigeura T, Tashiro T, et al. Natural killer T cell-targeted immunotherapy mediating long-term memory responses and strong antitumor activity. *Front Immunol*. 2017;8:1206.
- Smyth MJ, Thia KY, Street SE, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med*. 2000;191:661-668.
- Dellabona P, Casorati G, de Lalla C, Montagna D, Locatelli F. On the use of donor-derived iNKT cells for adoptive immunotherapy to prevent leukemia recurrence in pediatric recipients of HLA haploidentical HSCT for hematological malignancies. *Clin Immunol*. 2011;140:152-159.
- Nieda M, Nicol A, Koezuka Y, et al. TRAIL expression by activated human CD4⁺V α 24NKT cells induces in vitro and in vivo apoptosis of human acute myeloid leukemia cells. *Blood*. 2001;97:2067-2074.
- Takahashi T, Chiba S, Nieda M, et al. Cutting Edge: Analysis of Human V α 24⁺CD8⁺ NK T Cells Activated by α -Galactosylceramide-Pulsed Monocyte-Derived Dendritic Cells. *J Immunol*. 2002;168:3140-3144.
- Wingender G, Krebs P, Beutler B, Kronenberg M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol*. 2010;185:2721-2729.
- Yamada D, Iyoda T, Vizcardo R, et al. Efficient regeneration of human V α 24⁺ invariant natural killer T cells and their anti-tumor activity in vivo. *Stem Cells*. 2016;34:2852-2860.
- Consonni M, de Lalla C, Bigi A, Dellabona P, Casorati G. Harnessing the CD1 restricted T cell response for leukemia adoptive immunotherapy. *Cytokine Growth Factor Rev*. 2017;36:117-123.
- Kawano T, Nakayama T, Kamada N, et al. Antitumor cytotoxicity mediated by ligand-activated human V α 24 NKT cells. *Cancer Res*. 1999;59:5102-5105.
- Gansuvd B, Hagihara M, Yu Y, et al. Human umbilical cord blood NK T cells kill tumors by multiple cytotoxic mechanisms. *Hum Immunol*. 2002;63:164-175.
- Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8⁺ T cells by a flow cytometric assay for degranulation. *J Immunol Methods*. 2003;281:65-78.
- Aktas E, Kucuksezzer UC, Bilgic S, Erten G, Deniz G. Relationship between CD107a expression and cytotoxic activity. *Cell Immunol*. 2009;254:149-154.
- Mise N, Takami M, Suzuki A, et al. Antibody-dependent cellular cytotoxicity toward neuroblastoma enhanced by activated invariant natural killer T cells. *Cancer Sci*. 2016;107:233-241.

23. BrycesonYT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood*. 2006;107:159-166.
24. LongEO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol*. 2013;31:227-258.
25. RenJ, Liu X, Fang C, Jiang S, June CH, Zhao Y. Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. *Clin Cancer Res*. 2017;23:2255-2266.
26. BendelacA, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol*. 2007;25:297-336.
27. MotohashiS, Nagato K, Kunii N, et al. A phase I-II study of α -galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol*. 2009;182:2492-2501.
28. MatsudaM, Ono R, Iyoda T, et al. Human NK cell development in hIL-7 and hIL-15 knockin NOD/SCID/IL2rgKO mice. *Life Sci Alliance*. 2019;2:e201800195.
29. KuylentiernaC, Bjorkstrom NK, Andersson SK, et al. NKG2D performs two functions in invariant NKT cells: direct TCR-independent activation of NK-like cytotoxicity and co-stimulation of activation by CD1d. *Eur J Immunol*. 2011;41:1913-1923.
30. SharmaA, Lawry SM, Klein BS, et al. LFA-1 ligation by high-density ICAM-1 is sufficient to activate IFN- γ release by innate T lymphocytes. *J Immunol*. 2018;201:2452-2461.
31. Silva-SantosB, Serre K, Norell H. $\gamma\delta$ T cells in cancer. *Nat Rev Immunol*. 2015;15:683-691.
32. MoritaCT, Beckman EM, Bukowski JF, et al. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human $\gamma\delta$ T cells. *Immunity*. 1995;3:495-507.
33. VavassoriS, Kumar A, Wan GS, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human $\gamma\delta$ T cells. *Nat Immunol*. 2013;14:908-916.
34. GajewskiTF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol*. 2013;14:1014-1022.
35. WajchmanHJ, Pierce CW, Varma VA, Issa MM, Petros J, Dombrowski KE. Ex vivo expansion of CD8⁺CD56⁺ and CD8⁺CD56⁻ natural killer T cells specific for MUC1 mucin. *Cancer Res*. 2004;64:1171-1180.
36. RauletDH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol*. 2003;3:781-790.
37. SwannJB, Uldrich AP, van Dommelen S, et al. Type I natural killer T cells suppress tumors caused by p53 loss in mice. *Blood*. 2009;113:6382-6385.
38. MorrisES, MacDonald KP, Rowe V, et al. NKT cell-dependent leukemia eradication following stem cell mobilization with potent G-CSF analogs. *J Clin Invest*. 2005;115:3093-3103.
39. CroweNY, Smyth MJ, Godfrey DI. A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med*. 2002;196:119-127.
40. ChucAEN, Cervantes LAM, Retiguin FP, Ojeda JV, Maldonado ER. Low number of invariant NKT cells is associated with poor survival in acute myeloid leukemia. *J Cancer Res Clin Oncol*. 2012;138:1427-1432.
41. KimTW, Park SS, Lim JY, et al. Predictive role of circulating immune cell subtypes early after allogeneic hematopoietic stem cell transplantation in patients with acute leukemia. *Int J Stem Cells*. 2018;12:73-83.
42. ChaidosA, Patterson S, Szydlo R, et al. Graft invariant natural killer T-cell dose predicts risk of acute graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. *Blood*. 2012;119:5030-5036.
43. SchneidawindD, Baker J, Pierini A, et al. Third-party CD4⁺ invariant natural killer T cells protect from murine GVHD lethality. *Blood*. 2015;125:3491-3500.
44. WolfBJ, Choi JE, Exley MA. Novel Approaches to Exploiting Invariant NKT Cells in Cancer Immunotherapy. *Front Immunol*. 2018;9:384.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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