

Immune synapse molecules knockout in induced pluripotent stem cells enables broad NK cell resistance in T cell progeny

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Abstract

Introduction

Allogeneic iPSCs provide a potential source for regenerative T cell therapies, yet their clinical application remains limited by immune rejection driven by human leukocyte antigen (HLA) incompatibility. Knockout of β 2-microglobulin (B2M) eliminates HLA class I expression and protects against CD8⁺ T cell-mediated killing, but paradoxically provokes natural killer (NK) cell activation via the “missing-self” response. Existing approaches seek to enhance inhibitory signaling or dampen activating signals, yet these approaches achieve only partial NK evasion due to the extraordinary heterogeneity of NK receptor repertoires.

Methods

We developed a broader and more universal immune evasion strategy by targeting immune synapse (IS) adhesion molecules that critical for NK–target cell engagement. Building on our previously reported hypoimmunogenic iPSC-derived T cell (iT cell) platform (B2M^{KO}CIITA^{KO}PVR^{KO} with HLA-E overexpression), we engineered double-knockout (dKO) iT cells lacking CD54 (ICAM-1) and CD58 (LFA-3) using CRISPR/Cas9-mediated gene editing. These two ligands play key roles in stabilizing NK–target immunological synapses.

Results

Functionally, dKO iT cells exhibited marked resistance to NK cell–mediated cytotoxicity both in vitro and in vivo in human IL15 transgenic mouse model, while maintaining iT cell cytotoxic effector functions. By disrupting the physical interface required for NK engagement, this approach provides broad protection against diverse NK cell subsets and complements existing HLA-focused immune evasion strategies.

Conclusions

Our findings establish a potentially versatile platform for generating universal, NK-resistant iT cells and advance the translational potential of iPSC-based immunotherapies.

Keywords

iPSCs; Gene modification; Immune synapses; T cell therapy.

Abbreviations

HLA, Human leukocyte antigen; B2M, β 2-microglobulin; NK, Natural killer; IS, Immune synapse; induced pluripotent stem cells, iPSCs; iT cell, iPSC-derived T cell; dKO, double-knockout; CARs, chimeric antigen receptors; CDS, coding sequences; IVT, in vitro transcription; iHPCs, iPSC-derived hematopoietic progenitor cells; gRNAs, guide RNAs.

1. Introduction

Autologous primary T cells engineered with chimeric antigen receptors (CARs) have demonstrated substantial clinical success in the treatment of lymphoma, leukemia, and multiple myeloma¹². However, its widespread application is limited by high cost and time-intensive procedures³⁴. Compared to autologous approaches, allogeneic iT cells represent a promising off-the-shelf option, as clinically applicable and scalable iT cells have been generated from iPSCs⁵. In addition, the clonal expandability of iPSCs makes them well suited for precise gene editing⁶, allowing for the development of immune-evasive cell products to overcome HLA incompatibility–driven rejection⁷.

HLA-focused immune evasion strategies, such as simultaneous knockout of B2M and CIITA, essential for HLA-I or -II expression, can help evade killing by CD8 or CD4 T cells⁸. However, the absence of HLA class I molecules triggers NK cell activation via the “missing-self” response, resulting in elimination of B2M-deficient iPSC derived cells.

To overcome this, several strategies to evade NK cell responses have been explored, including knocking out B2M and single-chain HLA-E transduction into human PSCs⁹, selective retention of HLA-C while deleting HLA-A/B¹⁰, co-expression of CD47 with B2M/CIITA knockout¹¹, co-expression of synthetic SIRPα checkpoint engager and truncated CD64¹² and PVR knockout combined with HLA-E expression¹³. However, NK cells are highly heterogeneous, with substantial donor-specific variation in receptor expression. It has been reported that a single donor may possess up to 6,000 distinct NK cell phenotypes¹⁴. This diversity makes it challenging to achieve broad immune evasion by targeting individual activating or inhibitory pathways.

We confirmed this concern by evaluating our previously reported hypoimmunogenic iT cell (parental iT cell) platform. Parental iT cells largely evaded recognition by resting NK cells over a short period. However, evasion from IL-15–activated NK cells was not consistently effective, particularly after prolonged culture. Given that inflammatory cytokines such as IL-15 are abundant in clinical transplantation environments¹⁵, these findings highlighted a critical limitation of current HLA-focused approaches.

An alternative strategy is to disrupt IS formation—the physical interface required for NK-mediated killing. NK activation depends on cell–cell contact stabilized by adhesion receptors, including LFA-1 and CD2¹⁶, which bind their ligands CD54 and CD58. CRISPR-based screens have identified CD54 and CD58 as essential for NK-mediated killing of HLA-I-deficient tumor cells¹⁷.

Recent studies have explored double knockout (dKO) of CD54 and CD58 in B2M^{KO}CIITA^{KO} iPSC-derived NK cells, demonstrating reduced susceptibility to NK-mediated cytotoxicity¹⁸. However, these strategies have not been evaluated in the context of iT cells, and their potential additive benefit to existing hypoimmunogenic iT cell platforms remains unknown.

In this study, we found that iT cells derived from our previously established hypoimmunogenic parental iPSCs¹³ expressed uniformly high levels of CD54 and CD58, suggesting that CD54 and CD58 may remain a key route of NK activation.

We then generated single-knockout (CD54^{KO} or CD58^{KO}) and double-knockout (CD54^{KO} CD58^{KO}; dKO) iPSCs, and subsequently differentiated them into iT cells. Functional assays revealed that CD54/CD58 dKO iT cells exhibited significantly enhanced resistance to IL-15–activated NK cell cytotoxicity, outperforming previously reported dKO strategies in

iPSC-NK models. Furthermore, in human IL-15 transgenic NSG mice, dKO iT cells displayed prolonged in vivo persistence under strong NK pressure, compared to parental iT cells.

Importantly, the genetic modifications did not impair developmental potential into iT cells. At the T cell stage, CAR based recognition and tumor cytotoxicity remains unaffected.

Together, these findings establish CD54/CD58 double-knockout as a robust and broadly effective NK cell evasion strategy. By targeting IS formation rather than focusing solely on individual NK receptor pathways, this approach expands the utility of hypoimmunogenic iPSCs and provides a universal platform for generating durable, allogeneic, NK-resistant iT cells.

2. Methods:

2.1. Mice

hIL-15-NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} Tg(IL15)1Sz/SzJ) were obtained from The Jackson Laboratory. Age- and sex-matched mice were randomly assigned to experimental groups. All animals were maintained under specific pathogen-free conditions with controlled temperature, humidity, and a 12-hour light/dark cycle. All procedures were approved by the institutional animal care and use committee of Kyoto University and conducted in accordance with its ethical guidelines.

2.2. Cells

B2M KO CIITA KO HLA-E O/E PVR KO (tKO/E) human iPSC line from TKT3v1-7 iPSCs were established in our lab and used as parental iPSCs in this study. Human iPSC lines were cultured with StemFit AK02N media and iMatrix-511. Primary NK cells (CD3 neg CD56 dim bright) were sorted from PBMC, which isolated from healthy donors and cultured with 10% FBS DMEM medium, containing 10 ng ml⁻¹ IL-7 (PeproTech), 5 ng ml⁻¹ IL-15 (PeproTech). PBMCs were obtained from healthy volunteers. NALM-6 cells were a gift from Kyowa Hakko Kirin, originally purchased from American Type Culture Collection and cultured with 10% FBS RPMI medium. Wild-type K562 cells were purchased from the JCRB Cell Bank (Tokyo, Japan) and cultured with 10% FBS RPMI medium.

2.3. Guide RNAs (gRNA) synthesis and gene modifications in iPSCs

The coding sequences (CDS) of CD54 and CD58 were identified using BLAST in PubMed. gRNAs targeting exon 1 of CD54 (CGCACTCCTGGTCCTGCTCG) and CD58 (GGCCCTGGGGGTCCTCAGCG) were designed using CRISPOR. DNA templates encoding the gRNAs were synthesized and transcribed in vitro. To remove residual DNA, 1 µl TURBO DNase was added and incubated at 37 °C for 15 minutes. The RNA was then purified using the RNeasy MinElute Cleanup Kit. Purified in vitro transcription (IVT) products were stored at -80 °C until use. iPSCs (0.3 × 10⁶ cells) were transfected with a ribonucleoprotein complex of 5 µg recombinant Cas9 (IDT, cat. no. 1074181, or Thermo Fisher cat. no. A36498) and 1.25 µg of in vitro transcribed gRNA in 20 µl Primary P4 buffer and the electroporation condition program CA-137 using 4D-Nucleofector (Lonza). After electroporation, the cells were transferred to 9.6 cm² iMatrix511-coated 6-well plates and grown in AK02 medium containing the ROCK inhibitor Y-27632. The cells were analyzed by FACS on day 7 after culturing.

2.4. Sub-cloning and genotyping for gene edited iPSCs

Genome-edited iPSCs were cultured for 7 days and stained by CD54 and CD58 antibody. Then each single iPSC of the desired population was sorted by FACS AriaII (BD) into a well of iMatrix-511-coated 24-well plates and grown in AK02N medium containing ROCK inhibitor

Y-27632. 7days after single cell sorting when colony size reached greater than 1 mm² in diameter, then cells were passaged in a well of iMatrix-511-coated 6-well plate. After reaching confluency in the plate, half of the cells were frozen for cryogenic preservation and the other half were harvested for the isolation of genomic DNA. Genomic DNA was used for PCR and Sanger sequencing to analyze indel patterns.

2.5. Flow cytometry analysis

iPSCs and iT cells were prepared in a single cell suspension. The staining and washing steps were carried out in PBS containing 2% FBS (Biosera, cat. no. FB-1365). For staining, iPSCs and iT cell suspensions were incubated with 50–100µl of diluted antibodies for 20–30 min on ice. After adding propidium iodide (Invitrogen, cat. no. P3566), the samples were analyzed on a FACS Aria II (BD), and the data were processed using FlowJo software (v.9.9.6 or 10.5.3). The antibodies used in this study are listed in Supplementary Table 1.

2.6. NK cells sorting

NK cells from peripheral blood mononuclear cells. NK cells constitute 5–20 % of PBMC. To obtain NK cells, CD3+ cells are depleted and CD56+ cells selected. After isolation, NK were expanded with autologous PBMC as feeders to generate functional active NK cells, then stimulated with 2 µg/ml PHA (Sigma) and supported with irradiated PBMC feeders in α-MEM containing 10 ng/ml IL-7 and 5 ng/ml IL-15 (PeproTech).

2.7. T-cell differentiation from iPSCs

3×10⁶ iPSCs were seeded into an ultra-low attachment 6-well plate (Corning, Cat. No. 3471) and cultured with StemFit AK02N medium (Ajinomoto) and 10 mM ROCK inhibitor Y-27632 (Wako, Cat. No. 251-00514) in a 5% O₂, 5% CO₂ incubator for 2 days. From day 2, the medium was changed to StemPro-34 (Thermo Fisher Scientific, Cat. No. 10639011) with 50 ng/mL bFGF (Oriental Kobo, Cat. No. 47107000), 50 ng/mL VEGF (R&D Systems, Cat. No. 293-VE-050/CF), 1× ITS (Thermo Fisher Scientific, Cat. No. 41400-045), 2mM GlutaMax-I (Thermo Fisher Scientific, Cat. No. 35050061), 50 mg/mL L-ascorbic acid 2-phosphate. T-cell differentiation was induced by culturing iPSC-derived hematopoietic progenitor cells (iHPCs) on rhDL4-coated plates. To prepare the coating, a rhDL4/Fc chimera protein solution (10 µg/ml, Sino Biological) was mixed with retronectin (10 µg/ml, TAKARA, Japan), and 120 µl of the mixture was added to each well of a 48-well plate. Plates were incubated overnight at 4°C. The coating solution was removed immediately before cell seeding. On day 14 of differentiation, EBs were collected and filtered through a cell strainer to remove intact EBs and enrich for iHPCs. A total of 10,000 iHPCs were seeded into each DL4-coated well containing T-cell differentiation medium. The medium consisted of αMEM (Thermo Fisher Scientific) supplemented with 15% FBS (Corning), 1× ITS-G, 55 µM 2-mercaptoethanol, 50 µg/ml ascorbic acid-2-phosphate, 2 mM GlutaMAX, 50 ng/ml rhSCF, 50 ng/ml rhTPO, 50 ng/ml rhIL-7, 50 ng/ml FLT3L, 30 nM rhSDF-1α (PeproTech), and 15 µM SB203580 (Tocris Bioscience). Half of the medium was replaced every other day to maintain cytokine levels and nutrient balance. To support continued differentiation and prevent over-confluence, cells were transferred to fresh DL4-coated plates on days 7 and 14. On day 7 and day 14, cell density was adjusted to 1×10⁵ cells/well. On day 21, differentiated cells were collected and analyzed by flow cytometry.

2.8. Anti-CD19 iCAR-T cell generation

Anti-CD19 iCAR-T cells were generated using a retrovirus-mediated transduction system.

Retroviral particles were loaded onto RetroNectin-coated 24-well plates and centrifuged at 2,000g for 2 h. iT cells were preactivated with PHA-stimulated PBMCs for 3 days, then seeded onto the virus-coated plates and centrifuged at 300g for 5 min to facilitate transduction. After 14 days of culture, iCAR-T cells were sorted by FACS. The sorted cells were subsequently restimulated with PHA-stimulated PBMCs to expand cell numbers.

2.9. Luciferase-based cytotoxicity assay

Co-cultures were incubated in 96-well plates for 48h at 37°C. For iT cell survival analysis, cells were seeded at 2×10^4 cells/ml in α -MEM supplemented with 10 ng/ml IL-7 and 5 ng/ml IL-15 (PeproTech), and co-cultured with IL-15-activated NK cells at an E:T ratio of 1:1 in 96-well plates for 48h under the same conditions. After incubation, D-luciferin (100 μ g/ml) was added to each well and incubated for 10 min. Luminescence was measured using a plate reader. Survival was calculated as: $\text{Survival (\%)} = (\text{RLU from effector} + \text{target well}) / (\text{RLU from target-only well}) \times 100$. Percent killing was calculated as: $\text{Killing (\%)} = 100 - \text{Survival (\%)}$.

2.10. Counting bead-based cytotoxicity assay

To evaluate the cytotoxic activity of iCAR-T cells against CD19-expressing Nalm6 cells, target cells were seeded at 2×10^4 cells/ml in α -MEM supplemented with 10 ng/mL IL-7 and 5 ng/mL IL-15 (PeproTech). iCAR-T cells were added at varying effector-to-target (E: T) ratios (10:1, 5:1, 2.5:1, 1.25:1) in a total volume of 200 μ l per well in a 96-well plate. Co-cultures were incubated for 5 h at 37°C. Following incubation, 3,000 counting beads (C36950, Invitrogen) were added to each well. Samples were analyzed by flow cytometry, and data acquisition was stopped after 1,000 beads were recorded per well to standardize event collection. Absolute cell numbers were quantified based on bead counts.

Specific cytotoxicity was calculated using the following formula: $\text{Specific killing (\%)} = [1 - (\text{number of Nalm6 cells co-cultured with iCAR-T cells} / \text{number of Nalm6 cells cultured alone})] \times 100$.

To evaluate iT cell survival in the presence of resting NK cells, target cells were seeded at 1×10^5 cells/mL in RPMI medium supplemented with 10% FBS. NK cells were added at an E:T ratio of 1:1 in a total volume of 200 μ l per well in a 96-well plate. Co-cultures were incubated for 72 h at 37°C. For the cell count assay, at least 2,000 counting beads from absolute counting tubes (BD, 340334) were acquired per sample. Flow cytometry data acquisition was stopped after 1,000 beads were recorded per well to standardize event collection. Absolute cell numbers were quantified based on bead counts.

Specific cytotoxicity was calculated using the following formula:

$\text{Specific killing (\%)} = [1 - (\text{number of iT cells co-cultured with NK cells} / \text{number of iT cells cultured alone})] \times 100$.

2.11. CD107a assay

NK cells were co-cultured with iT cells at a 1:1 ratio in a 96-well Flat-plate for 4h in 200 μ l in α -MEM supplemented with 10 ng/mL IL-7 and 5 ng/mL IL-15 (PeproTech), with 2 μ M monensin (Biolegend, 420701), and 1 μ l anti-CD107a antibody (BioLegend, cat. no. 328626 Clone H4A3). Finally, the percentage of CD107a-expressing NK cells was measured by flow cytometry.

2.12. Conjugate formation assay

NK cells were co-cultured with iT cells at a 1:1 ratio in a 96-well flat-bottom plate for 4 h in 200 μ l α -MEM supplemented with 10 ng/mL IL-7, 5 ng/mL IL-15 (PeproTech), 2 μ M monensin

(BioLegend, 420701), and 1 μ l anti-HLA-ABC antibody (BioLegend, cat. no. 311410, clone W6/32). The percentage of conjugates was measured by flow cytometry.

2.13. In vivo assessment of NK cell immunoreactivity against iT cells

To evaluate the immune reactivity of NK cells against iT cells, 5×10^5 luciferase-expressing iT cells were injected intraperitoneally into 12-week-old female hIL-15 NSG mice, with or without 5×10^5 NK cells. Each group included four mice. IVIS imaging was performed on days 2, 3, 4, and 7 to evaluate cell persistence.

2.14. Statistical analysis

Number of samples of experiments are listed in the figure legends. All Data is presented as mean $\pm$ SD. Statistics were performed using Prism (GraphPad software). p-values were calculated by either two-tailed t-test for comparing two groups or two-way ANOVA with Tukey's multiple comparison test for more than three groups. $p < 0.05$ was considered significant.

2.15. OKT3 stimulation

To coat the culture plates, anti-human CD3 antibody (BioLegend, 317302) was diluted to 1 μ g/ml in D-PBS, and 110 μ l was added to each well of a 48-well plate. Plates were incubated overnight at 4°C. On the following day, cells were seeded at 2.6×10^5 cells/ml in α -MEM supplemented with 10 ng/ml IL-7, 5 ng/ml IL-15 (PeproTech), IL-12 p70 (Sigma, 50 ng/ml), IL-18 (MBL, 50 ng/ml) and IL-21 (PeproTech, 20 ng/ml).

2.16. CBA assay for quantification of cytokine secretion by iT Cells

To measure cytokine levels, 15 μ l of culture supernatant was collected from each sample. A standard curve ranging from 0 to 5000 pg/ml was prepared by serial dilution of the cytokine standard in Assay Diluent, with 15 μ l used per point. For each test, 1.3 μ l of IL-2 and IFN- γ capture beads were added, followed by 9 μ l of PE detection reagent and 16 μ l of PBS. Samples were incubated at room temperature for 3 hours. After incubation, 160 μ l of PBS was added to each tube prior to flow cytometry analysis. The expression levels were calculated using FCAP Array software.

3. Results

3.1. Parental hypoinmunogenic iT cells fail to evade NK-mediated cytotoxicity, especially under cytokine-driven activation.

To comprehensively evaluate the immune-evasive potential of our previously established hypoinmunogenic iT cells platform, we assessed the susceptibility of iT cells to NK cell-mediated cytotoxicity under two physiologically relevant conditions: (1) prolonged exposure to resting NK cells, and (2) challenge by cytokine-activated NK cells. We first isolated CD3⁺CD56⁺ NK cells from human PBMCs (Supplementary Fig. 1a) and co-cultured them with parental iT cells (Supplementary Fig. 1b). After 72 hours, > 40% lysis of iT cells was observed (Supplementary Fig. 1c), demonstrating that despite B2M/CIITA/PVR knockout and HLA-E overexpression, parental iT cells fail to evade NK-mediated killing during extended exposure.

Next, we assessed susceptibility to cytokine-activated NK cells. We compared three activation conditions—IL-7 + IL-2, IL-7 + IL-12, and IL-7 + IL-15—based on the known role of IL-7 in supporting CD56^{bright} NK survival¹⁹. NK cells were pre-activated for five days and subsequently co-cultured with parental iT cells (Supplementary Fig. 1d). Among these

conditions, while resting NK cells showed low cytotoxicity to parental iT cells, IL-7 + IL-15-activated NK cells displayed the strongest cytotoxic activity, inducing >80% iT cell lysis within 48 hours (Supplementary Fig. 1e). These results indicate that current hypoimmunogenic iT cells remain highly vulnerable to cytokine-driven NK cytotoxicity despite with HLA-E overexpression and PVR knockout.

We analyzed the expression of CD159a (NKG2A), the inhibitory NK receptor for HLA-E, as well as DNAM-1 and TiGiT, which are activating and inhibitory receptors, respectively, that interact with PVR, across NK cells from multiple healthy donors (Supplementary Fig. 2a). CD159a was uniformly expressed, suggesting that HLA-E overexpression may broadly engage inhibitory signaling in NK cells. In contrast, DNAM-1 was variably expressed, indicating that PVR knockout may not prevent recognition by all NK cells. Moreover, because NK cells express TiGiT, loss of PVR may also weaken TiGiT-mediated inhibitory signals, potentially reducing overall NK cell inhibition, particularly when NK cell were pre-activated.

Together, these results demonstrate that our previously engineered hypoimmunogenic iT cell platform provides short-term protection from NK-mediated cytotoxicity under resting conditions but fails to confer sufficient resistance against cytokine-activated NK cells or during prolonged culture.

3.2. Integrated receptor–ligand profiling identifies CD54, CD58 as targets in iT cells.

To develop next-generation engineering strategies for broad NK cell evasion, we hypothesized that residual IS formation contributes to the vulnerability of parental iT cells. Because NK activation relies on stable cell–cell contacts mediated by adhesion receptors, we first sought to identify candidate IS ligands whose disruption could broadly suppress NK-mediated cytotoxicity (Fig. 1a).

We analyzed the expression of LFA-1, CD2—the NK receptors for CD54, CD58—across NK cells from multiple healthy donors (Fig. 1b). Flow cytometry revealed near-universal (>95%) expression of the two receptors (Fig. 1c), indicating that NK activation pathways involving these ligands are highly conserved. This provided a strong rationale for selecting CD54 and CD58 as candidate targets for disrupting NK–target IS formation.

Next, we assessed CD54 and CD58 expression on parental iT cells. Flow cytometry revealed that parental iT cells displayed uniformly high expression of CD54, CD58, indicating that both of the two ligands are readily available for NK receptor engagement during IS formation.

Based on this, we selected CD54, CD58 as high-priority engineering targets and designed dKO iT cells to prevent IS formation and enhance NK evasion under both resting and cytokine-activated conditions.

3.3. Generation of adhesion molecule modified iPSCs

To dissect the individual roles of CD54 and CD58 in NK cell evasion, we used CRISPR/Cas9-mediated gene editing to generate a panel of adhesion molecule–deficient iPSC lines from our previously established hypoimmunogenic parental iPSCs. We first designed gRNAs targeting CD54, CD58 and introduced them individually or in combination using CRISPR/Cas9 editing (Fig. 2a). Three lines were successfully derived (Fig. 2b), clonally expanded, and validated. Flow cytometry of CD54 and Sanger sequencing of CD58 confirmed biallelic frameshift mutations at the targeted loci in iPSC (Fig. 2c, 2d). These protein-level knockouts were further validated in iT cells, based on our prior finding that CD54, CD58 are

consistently expressed in iT cells.

3.4. dKO iPSCs were successfully differentiated into iT cells

T cells represent one of the most clinically relevant and functionally specialized lineages derived from iPSCs. To evaluate whether adhesion molecule editing affects this critical lineage, we differentiated parental, CD54^{KO}, CD58^{KO} and double-knockout (dKO: CD54^{KO}CD58^{KO}) iPSCs into T cells using our previously established Notch-based protocol (Fig. 3a). On day 21, we assessed early T cell commitment by analyzing the proportion and number of CD4⁺CD8⁺ double-positive T cells. Flow cytometry confirmed the presence of CD4⁺CD8⁺ cells in both groups (Fig. 3b), with no significant differences in either percentage (Fig. 3c; Supplementary Fig. 3a) or absolute number (Fig. 3d), indicating that loss of CD54, CD58 does not impair initial T lineage specification.

We next evaluated later T cell maturation by stimulating the CD4⁺CD8⁺ cells with plate-bound OKT3 for 3 days, followed by a 4-day resting phase. On day 28, we analyzed single-positive CD8 $\alpha\beta$ ⁺ T cells (Supplementary Fig. 3b). Both parental and dKO groups successfully generated CD8 $\alpha\beta$ ⁺ T cells (Fig. 3e). Similar to the case of CD4⁺CD8⁺ cells, the dKO group showed no significant difference in CD8 $\alpha\beta$ ⁺ percentage compared to parental controls (Fig. 3f), with comparable total CD8 $\alpha\beta$ ⁺ cell numbers (Fig. 3g), indicating that the final T cell yield remains unchanged.

We further assessed whether dKO iT cells could expand and mature after extended stimulation. Following 14 days of expansion with PHA-stimulated PBMC and cytokine support, both groups retained CD8 $\alpha\beta$ ⁺ profiles (Fig. 3h), with comparable frequencies (Fig. 3i) and fold expansion rates (Fig. 3j), indicating similar proliferative capacity and maturation potential. We also included the HLA unedited WT iT cells for comparison and found that the proliferation capacity of the modified iT cell groups was comparable to that of the WT group (Supplementary Fig. 4a, 4b). To evaluate whether gene modifications impact cytokine secretion upon activation, iT cells were harvested on Day 10 following PHA/PBMCs expansion and stimulated with OKT3 for 24 hours. Cytokine levels in the supernatant, IFN- γ and IL-2, were then quantified. Compared to WT iT cells, cytokine secretion was not significantly altered in the gene-modified groups (Supplementary Fig. 4c, 4d).

To test the functionality of engineered iT cells, we transduced them with a CD19-specific CAR and evaluated cytotoxicity against CD19-expressing target cells (Fig. 4a). CAR expression was confirmed by flow cytometry (Fig. 4b). In a 5-hour co-culture assay (Fig. 4c; Supplementary Fig. 3c), iCAR-T cells derived from both parental and dKO lines exhibited potent and comparable lytic activity against Nalm6 cells, Raji cells and EBV-LCLs (Fig. 4d), confirming intact effector function.

3.5. dKO iT cells evade NK-mediated cytotoxicity by impairing IS formation in vitro and in vivo.

Given that CD54, CD58 are highly expressed on iT cells, we hypothesized that targeting these molecules could also reduce NK cell recognition and facilitate immune evasion. To test this, we co-cultured iT cells with cytokine activated NK cells for 48 hours (Fig. 5a). While nearly 80% of parental iT cells were eliminated, dKO iT cells showed about 30% cytotoxicity, indicating robust NK resistance (Fig. 5b). We next examined NK activation by measuring surface CD107a expression after 4 hours of co-culture (Fig. 5c). NK cells exposed to dKO iT cells showed significantly reduced CD107a expression compared to those co-cultured with

parental iT cells (Fig. 5d, 5e; Supplementary Fig. 3d), indicating blunted NK degranulation.

To investigate the mechanism of reduced NK activation, we analyzed IS formation by quantifying NK–T cell conjugates after 4 hours of co-culture (Fig. 5f). Flow cytometry revealed a significantly lower frequency of conjugate formation between NK cells and dKO iT cells compared to parental controls (Fig. 5g, 5h), suggesting that simultaneous knockout of CD54 and CD58 disrupts physical engagement between NK cells and their targets, thereby impairing IS formation.

We further tested whether this NK evasion phenotype persisted in vivo. Parental or dKO iT cells were injected intraperitoneally into hIL-15–NSG mice, either alone or together with activated NK cells (Fig. 6a, Supplementary Fig. 5a). Bioluminescence imaging was performed at days 2, 3, 4, and 7 post-injection (Fig. 6b). In the absence of NK cells (control group), both parental and dKO iT cells gradually declined, likely due to insufficient survival cytokines such as IL-7. In mice receiving NK cells, however, parental iT cells were rapidly eliminated, with luminescence nearly undetectable by day 2. In contrast, dKO iT cells maintained luminescence at levels comparable to the control group across day2 and day3. Quantification of relative survival (NK/control ratio) showed a significant advantage for dKO iT cells at day2, day3 and day7 (Fig. 6c), demonstrating that these cells effectively resist NK cell–mediated clearance in vivo.

Collectively, these findings establish that CD54/CD58-deficient iT cells resist NK cell–mediated cytotoxicity both in vitro and in vivo by limiting IS formation and reducing NK activation.

4. Discussion

The development of universal iT cell therapies offers a promising avenue toward off-the-shelf treatments for a wide range of hematologic and immune-related diseases. Nonetheless, the persistent challenge of host immune rejection, especially from NK cells, continues to limit their in vivo persistence and therapeutic efficacy—particularly in the context of HLA-edited hypoinmunogenic iT cells, where NK cells can still recognize and eliminate MHC-deficient targets via the “missing-self” response.

In this study, we present an immune evasion strategy based on the simultaneous knockout of two key adhesion molecules—CD54 and CD58—in our first-generation hypoinmunogenic iPSCs¹³. This approach impairs IS formation between NK cells and iT cells, thereby broadly suppressing NK activation and cytotoxicity and enabling improved cell survival both in vitro and in vivo in mouse model.

Unlike existing NK evasion strategies that focus on modulating inhibitory or activating receptor pathways—such as overexpressing HLA-E to inhibit NKG2A⁺ NK cells or knocking out PVR to avoid DNAM-1 recognition—our approach disrupts the physical interface required for NK–target engagement. CD54 and CD58 are canonical ligands for LFA-1 and CD2, respectively. By removing these essential surface ligands, we weaken the mechanical and molecular anchoring required for synapse formation, which is a shared prerequisite across NK subsets, thus achieving broad and donor-independent protection.

We found that knockout of either CD54 or CD58 reduced the sensitivity of iT cells to NK cell-mediated killing, as indicated by decreased CD107a expression on NK cells after co-culture. The effect of CD58 knockout was more pronounced than that of CD54. Maximal

resistance to NK-mediated killing was observed only in the double knockout (dKO) condition. These findings highlight the functional redundancy of adhesion molecules and demonstrate that simultaneous deletion is required to effectively disrupt the NK–target cell interaction. Importantly, knockout of adhesion molecules did not impair progenitor T cell differentiation. We further demonstrate that dKO cells can differentiate into CD8 $\alpha\beta$ ⁺ T cells and undergo expansion upon stimulation. In addition, the percentage of CD8 $\alpha\beta$ ⁺ cells was not reduced in the dKO group, suggesting that knockout of CD54 and CD58 did not impair adhesion-dependent TCR signaling during plate-bound OKT3 stimulation. Finally, our study highlights the potential of dKO iT cells to evade NK cell surveillance in vivo. These cells resisted clearance after adoptive transfer into IL-15–humanized mice, maintaining survival comparable to controls without NK cells. Notably, their cytotoxic function remained intact, and they retained the ability to kill tumor targets upon CAR transduction post coculture.

Despite these promising results, the removal of adhesion molecules could have unintended consequences on other cellular functions. For example, CD54 and CD58 also mediate cell migration²⁰²¹, endothelial adhesion²², and intercellular communication²³. Their absence could influence tissue homing, extravasation, or interactions with other immune cells, such as antigen-presenting cells (APCs)²⁴. Although we did not observe any gross defects in T cell differentiation or expansion, future studies should evaluate homing capacity, chemokine responsiveness, and in situ functionality of dKO cells in more physiological contexts.

To further improve clinical safety and flexibility, several translational enhancements can be envisioned. For instance, point mutations targeting only the NK receptor-binding domains of adhesion molecules could preserve their physiological roles while eliminating immune recognition. Moreover, incorporating suicide switches such as iCasp9²⁵ would provide a safeguard mechanism for rapid clearance of grafts in case of adverse events, a key requirement for regulatory and clinical translation.

Importantly, this work builds directly upon our previously established hypoimmunogenic iT cell platform, which combines disruption of HLA-I and HLA-II expression with enhanced NK cell inhibition through HLA-E overexpression and PVR knockout. By integrating adhesion molecule editing on top of this foundation, we introduce an additional layer of protection specifically targeting NK cell-mediated clearance—without compromising differentiation potential or effector function.

5. Conclusion

Together, our findings support a next-generation universal iT cell platform that combines genetic hypoimmunogenicity with structural NK evasion, and which can be further adapted to incorporate safety switches, lineage-specific enhancements, or even tumor-targeting modifications. This integrated strategy potentially enables the development of more durable, safe, and broadly applicable allogeneic iPSC-derived therapies.

Acknowledgement

We thank Ayako Kumagai, Ryohei Takada (Kyoto University) and Huaigeng Xu (UCSF) for their dedicated technical assistance. We further acknowledge the insightful research advice provided by Shoichi Iriguchi, Atsutaka Minagawa (Kyoto University), and Yohei Kawai (Shinobi), which greatly contributed to the progress of this study.

Contribution

J.Z. and B.W. designed the study. J.Z., B.W. and S.K. interpreted the data. J.Z., B.W., K.S., K.K., A.I., M.Y., T.I. performed the experiments. S.K. and B.W. supervised the study; J.Z., B.W. and S.K. wrote the manuscript.

Funding

This work was supported by the Japan Agency for Medical Research and Development (grant numbers: JP21am0101078, JP21am0401013, and JP21bm1004001). Japan Society for the Promotion of Science (22K15490)

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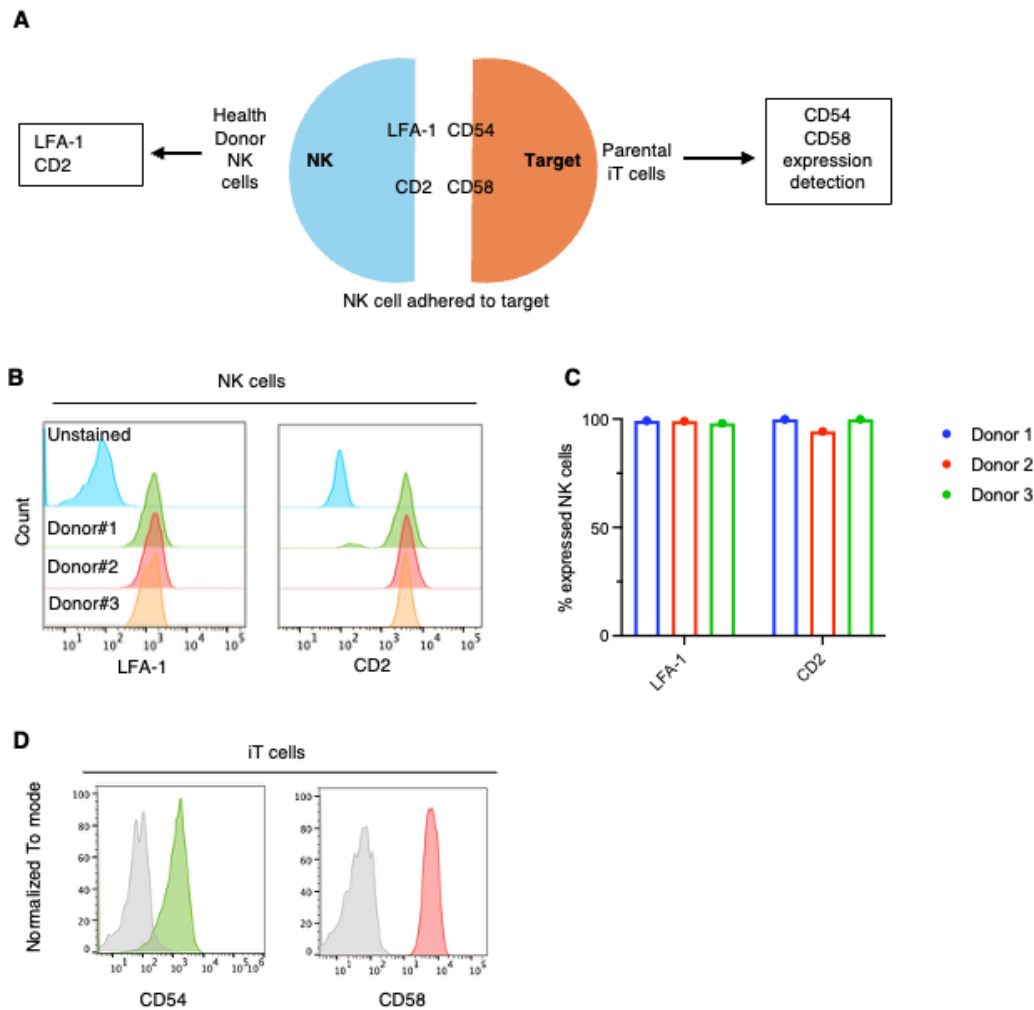


Figure 1 | Expression of common adhesion molecules on NK cells and iT cells.

A, Schematic overview of receptor–ligand interactions between NK cells derived from healthy donors and iT cells. **B**, Flow cytometry analysis of LFA-1 and CD2 expression on NK cells from three independent donors. **C**, Quantification of the proportion of NK cells expressing LFA-1 and CD2 from the same donors as in **B**. **D**, Flow cytometry analysis of CD54 and CD58, expression on iT cells.

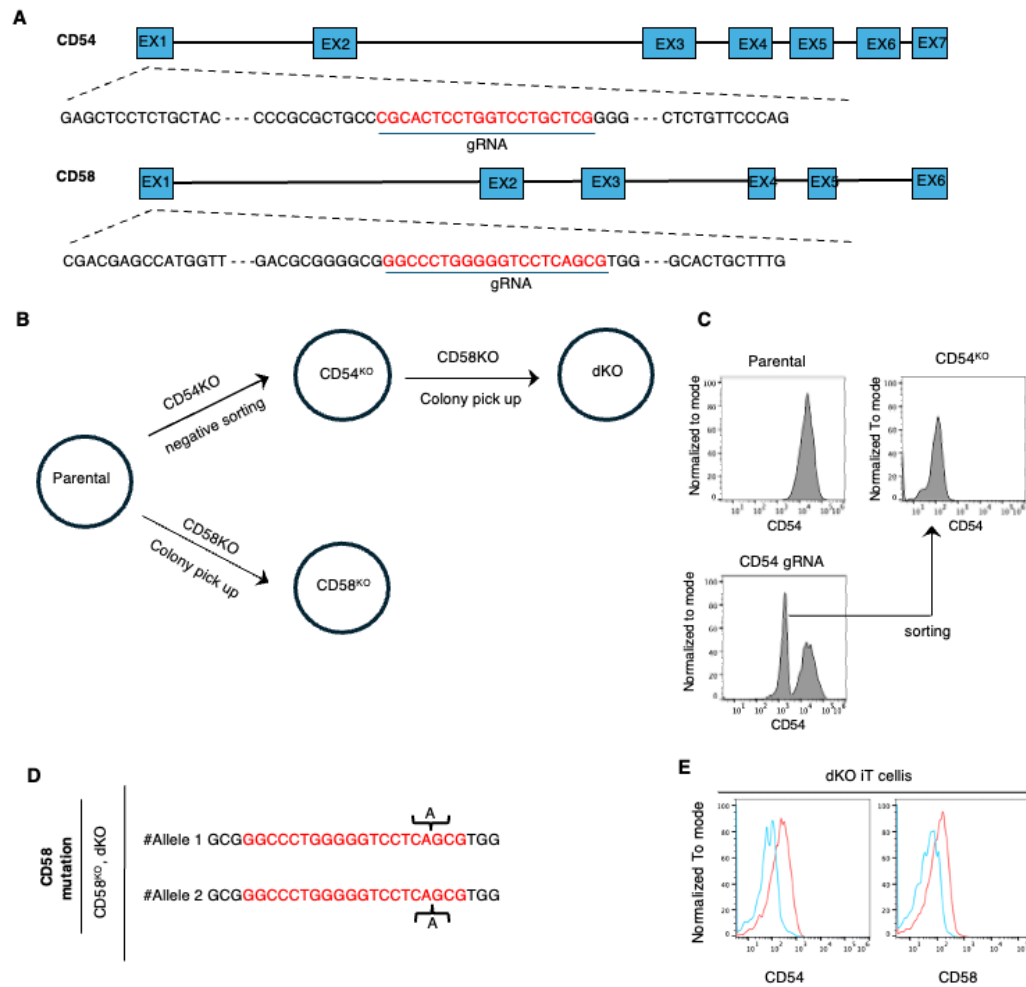


Figure 2 | CRISPR/Cas9-mediated genome editing of iPSCs.

A, Target sequences in exon 1 of CD54 and exon 1 of CD58, with gRNA binding sites highlighted in red. **B**, Workflow of the gene editing process in iPSCs. **C**, Flow cytometry analysis of CD54 expression in parental iPSCs, after genome editing, and following cell sorting. **D**, Representative nucleotide sequence alignment showing indel mutations in the CD58^{KO} clone. **E**, FACS analysis of CD54 and CD58 expression in iT cells differentiated from dKO iPSCs.

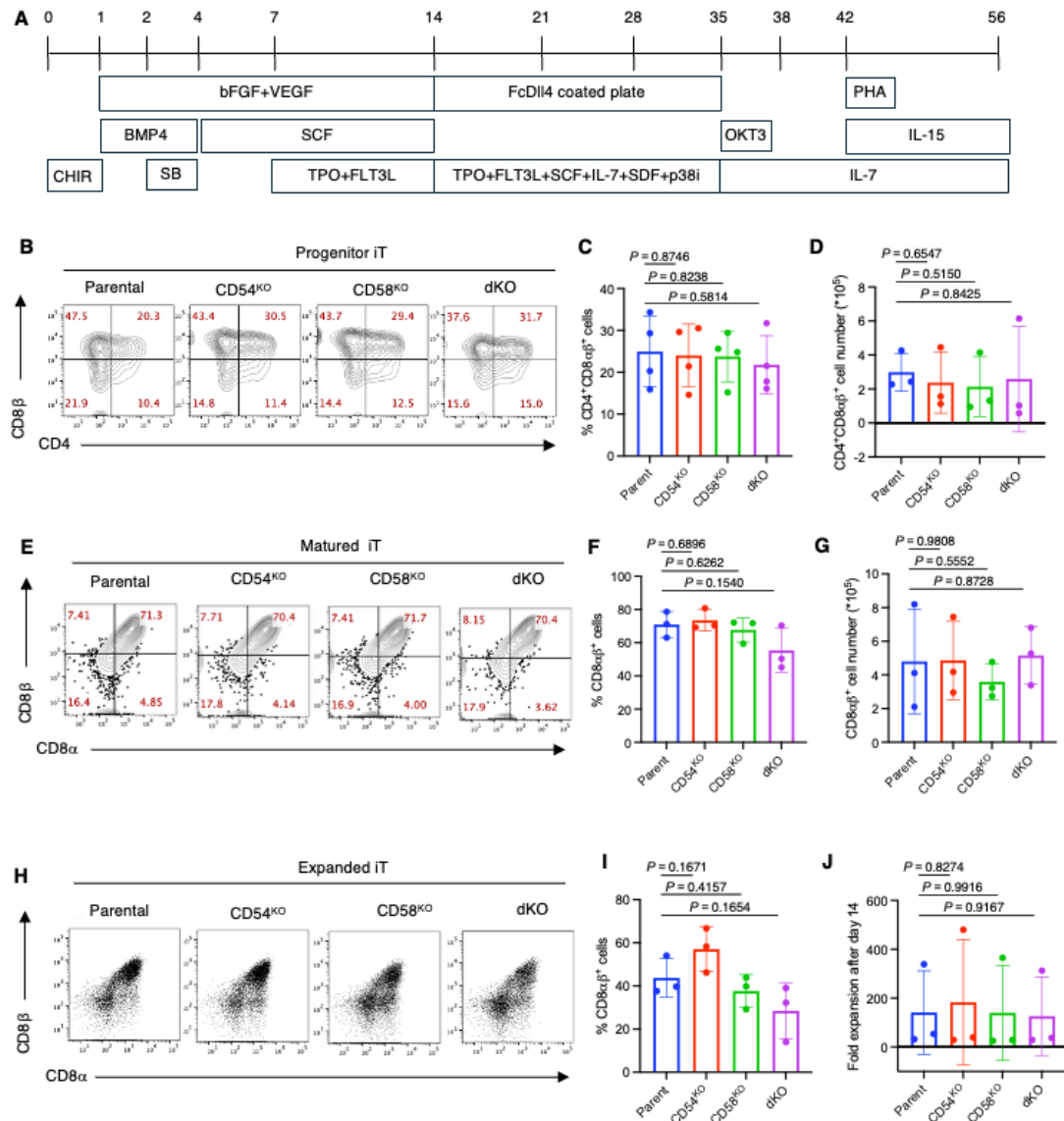


Figure 3 | Generation and functional characterization of iT cells. **A**, Schematic representation of the stepwise differentiation protocol used to generate T cells from iPSCs. **B**, Flow cytometry analysis of surface markers associated with CD4⁺CD8⁺ cells on day 21 of differentiation. **C**, Proportion of CD4⁺CD8⁺ cells on day 21. P values were determined using a two-tailed, unpaired t-test. Data are mean ± s.d. (n = 4 per group). **D**, Absolute number of CD4⁺CD8⁺ cells on day 21. P values were determined using a two-tailed, unpaired t-test. Data are mean ± s.d. (n = 3 per group). **E**, Flow cytometry analysis of surface markers associated with CD8αβ⁺ on day 28 of differentiation. **F**, Proportion of CD8αβ⁺ cells on day 28. P values were determined using a two-tailed, unpaired t-test. Data are mean ± s.d. (n = 3 per group). **G**, Absolute number of CD8αβ⁺ cells on day 28. P values were determined using a two-tailed, unpaired t-test. Data are mean ± s.d. (n = 3 per group). **H**, Flow cytometry analysis of CD8αβ⁺ cell surface markers on day 14 after expansion. **I**, Proportion of CD8αβ⁺ cells on day 14 post-expansion. P values were determined using a two-tailed, unpaired t-test. Data are mean ± s.d. (n = 3 per group). **J**, Fold expansion of iT cells over 14 days. P values were determined using a two-tailed, unpaired t-test. Data are mean ± s.d. (n = 3 per group).

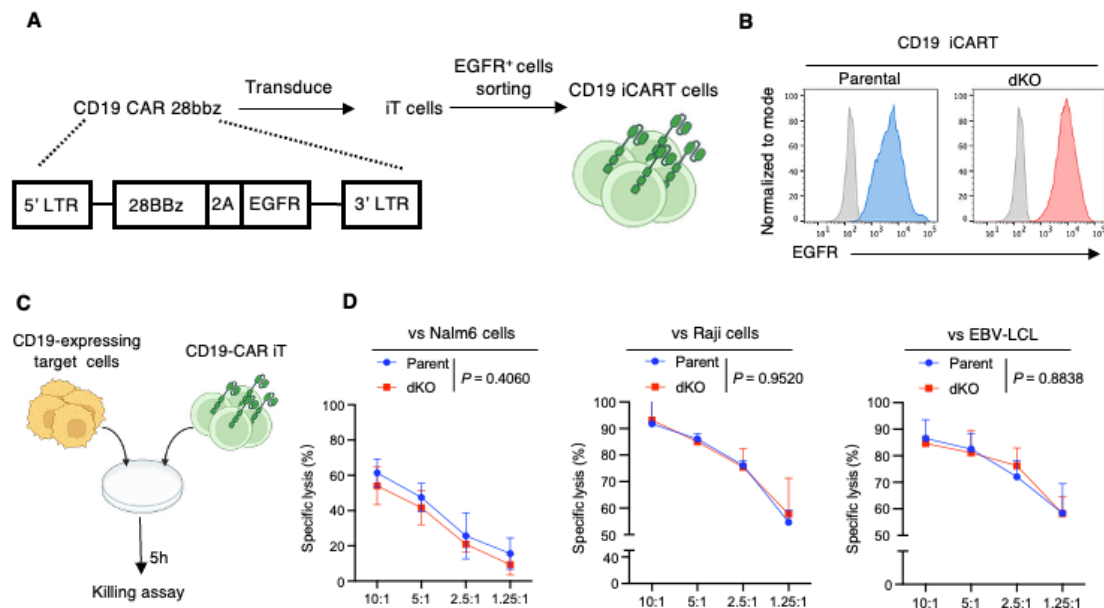


Figure 4 | Functional characterization of iT cells. **A**, Schematic representation of the generation and evaluation of iCAR-T cells. The RV-28BBz CAR construct enables co-expression of the CAR and truncated EGFR under the control of a single LTR promoter via a self-cleaving P2A sequence. LTR, long terminal repeat; P2A, Porcine teschovirus self-cleaving 2A sequence. **B**, Flow cytometry analysis of CD19 CAR expression in transduced iT cells. **C**, Schematic of the cytotoxicity assay. CD19 CAR-iT cells were co-cultured with CD19-expressing tumor cells for 5 hours. **D**, Specific lysis of Nalm-6 cell, Raji cells and EBV-LCL cells by CD19 iCAR-T cells. P values were determined using two-way ANOVA with multiple comparisons (n = 3 per group).

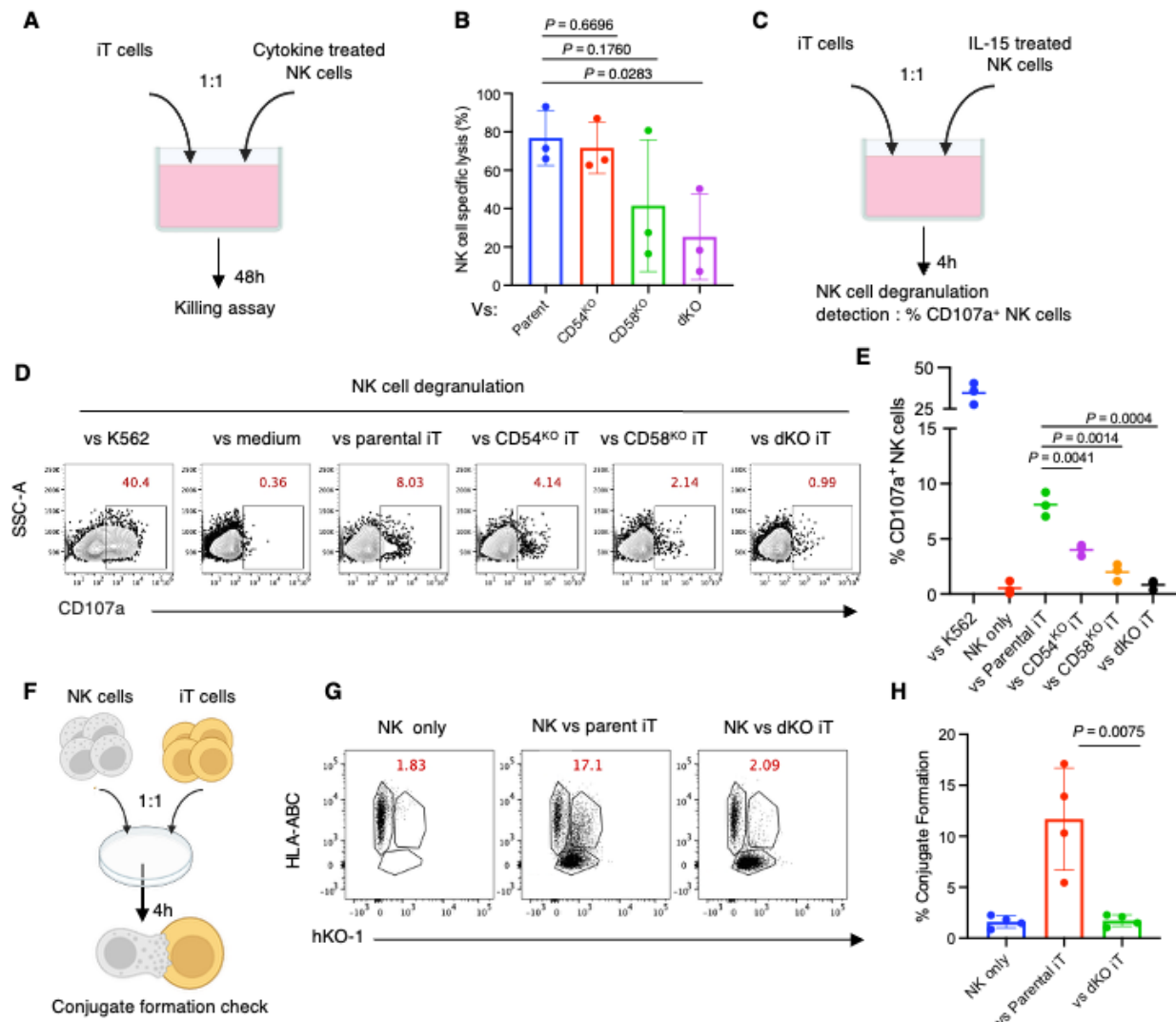


Figure 5 | Immunological characterization of iT cells. **A**, Experimental design of the immunogenicity assay. iT cells were co-cultured with cytokine-activated NK cells for 48 hours. **B**, Specific lysis of iT cells following co-culture with NK cells. P values were determined using a two-tailed, unpaired t-test. Data are mean $\pm$ s.d. ($n = 3$ per group; data represent three independent experiments using cells from 3 different donors (Donor #1, #3 and #4)). **C**, Schematic of the NK activation assay based on CD107a expression after 4 hours of co-culture. **D**, Flow cytometry analysis of CD107a expression on NK cells. Medium alone was used as a negative control; K562 cells served as a positive control. **E**, Quantification of CD107a⁺ NK cells. P values were determined using a two-tailed, paired Student's t-test. Data are mean $\pm$ s.d. ($n = 3$ per group; NK cells were isolated from three independent donors ((Donor #1, #2, #3)). **F**, Schematic of the conjugate formation assay performed after 4 hours of co-culture. **G**, Flow cytometry analysis of NK-iT cell conjugate formation. NK cells alone were used as a negative control. **H**, Quantification of conjugate formation. P values were determined using a two-tailed, unpaired t-test. Data are mean $\pm$ s.d. ($n = 4$ per group; NK cells were isolated from four independent donors (Donor #1, #2, #3, #4)).

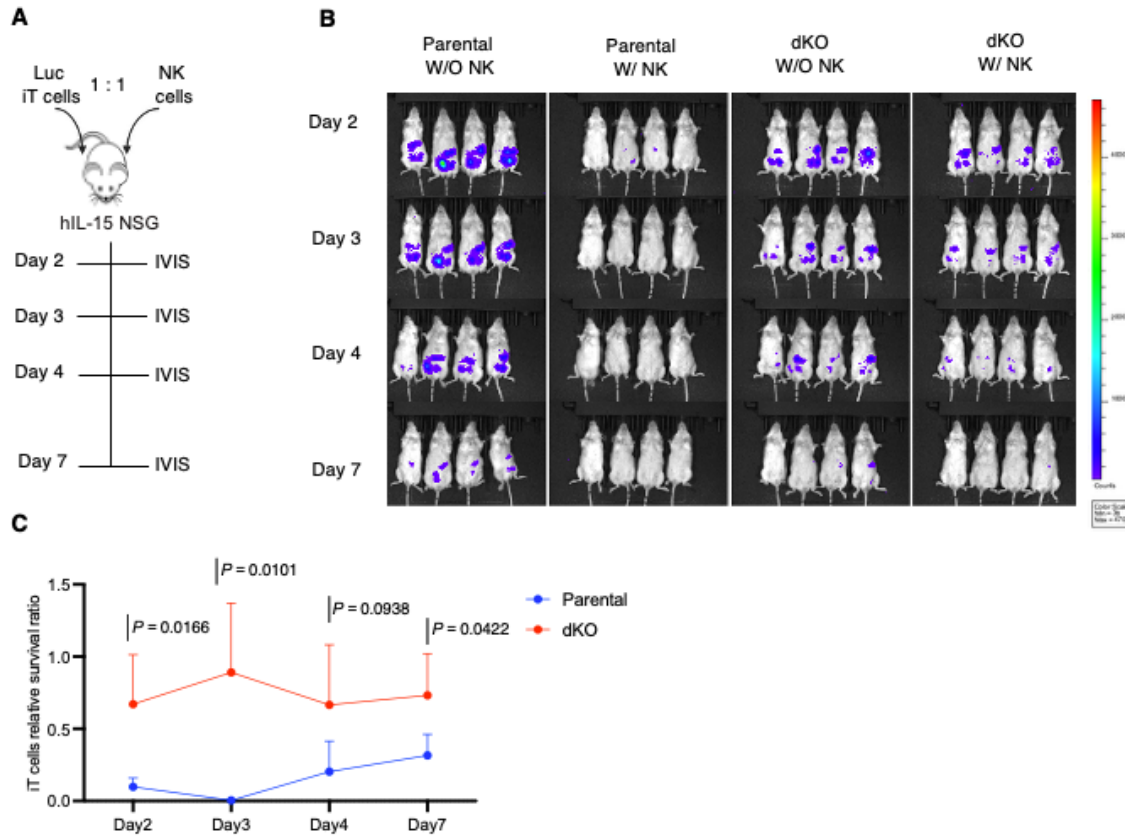
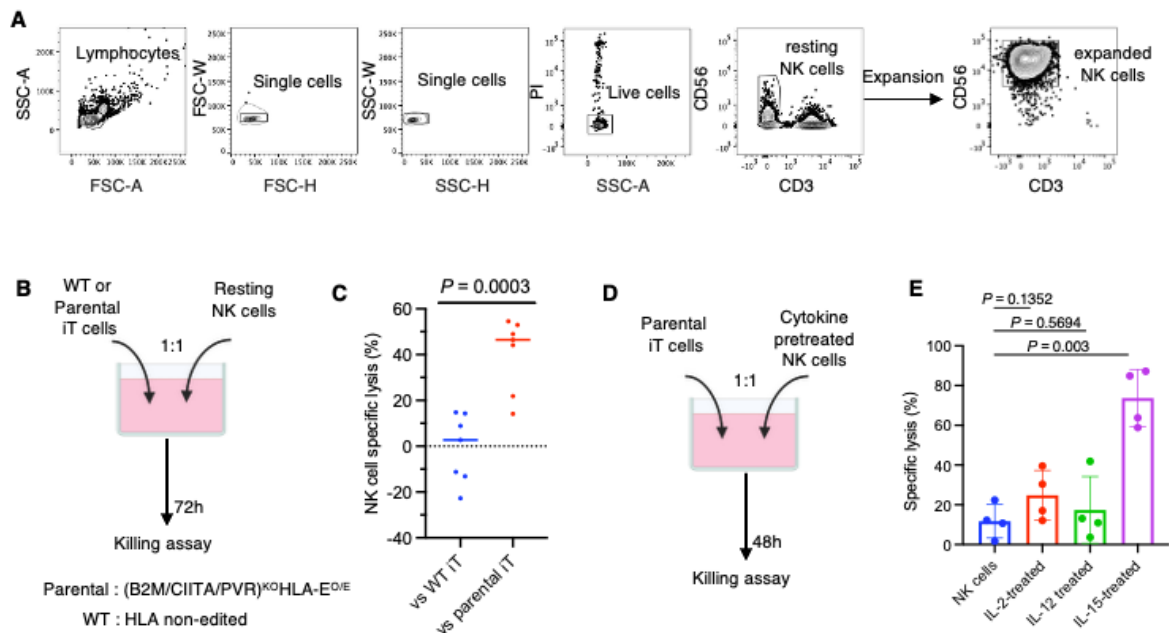


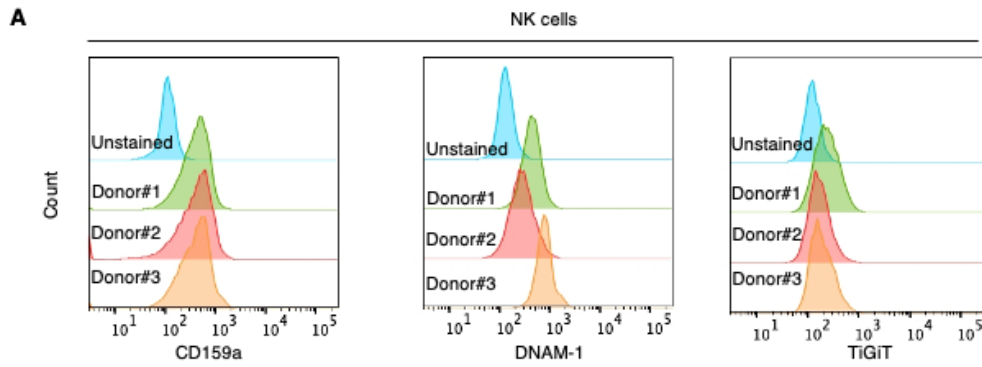
Figure 6 | In vivo assessment of hypoimmunogenic iT cells using an hIL-15-NSG mouse.

A, Experimental timeline. Human NK cells (donor #4) were mixed with parental or dKO iT cells at a 1:1 ratio, or with medium alone (control), and co-injected intraperitoneally into hIL-15-NSG mice. **B**, Bioluminescence imaging was performed at 2, 3, 4, and 7 days post-injection using IVIS. W/O = without; W/ = with. **C**, Quantification of total bioluminescence at the indicated time points. Relative survival ratios were calculated as the bioluminescence signal of iT cells co-injected with NK cells divided by the signal from iT cells injected without NK cells. P values were determined using a two-tailed, unpaired t-test. Data are mean $\pm$ s.d. (n = 4 per group)



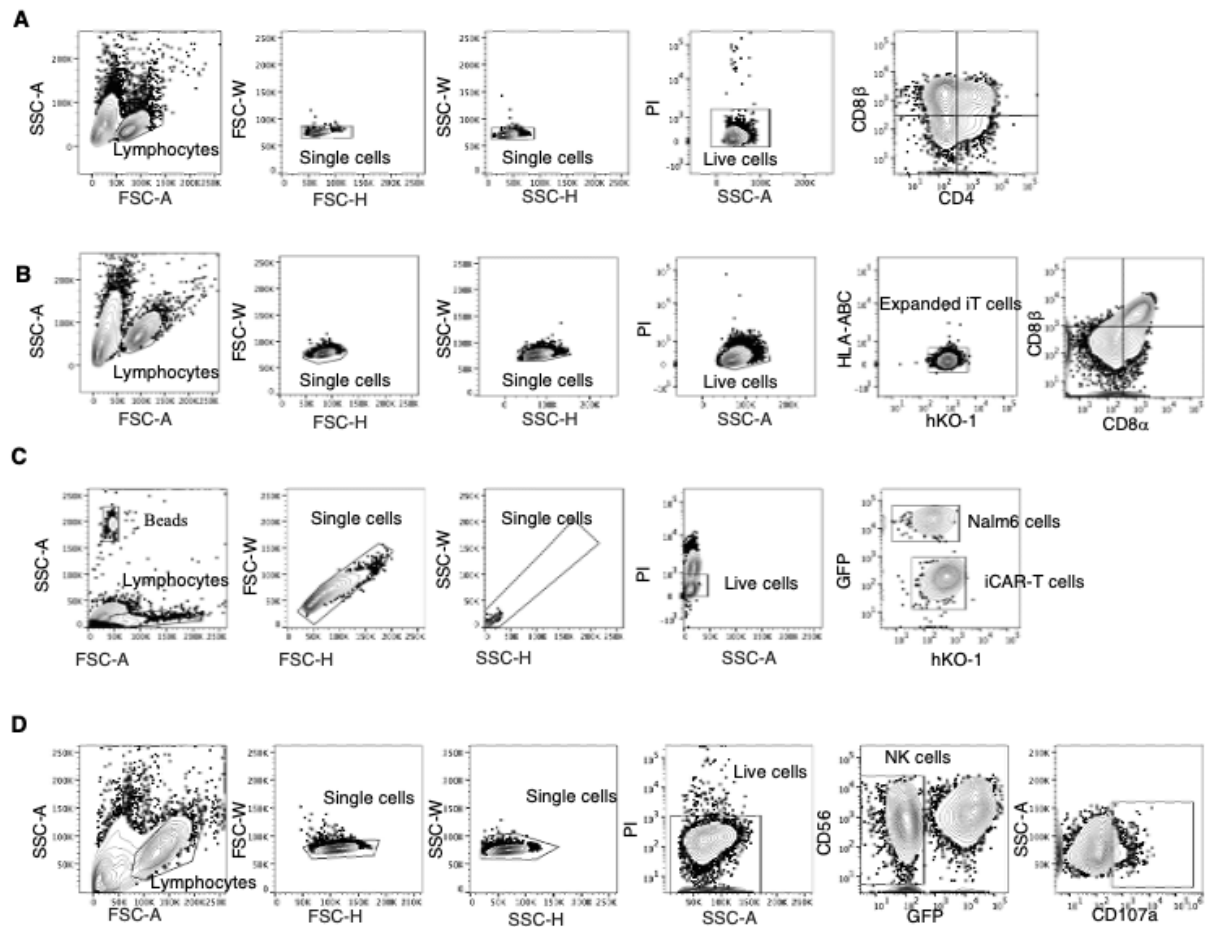
Supplementary Figure 1 | Limited immune evasion of current hypoiimmunogenic iPSC-derived cells.

A, CD56⁺CD3⁻ NK cells were sorted from PBMCs and expanded using PHA-stimulated PBMCs. **B**, Experimental design of the immunogenicity assay. Parental iT cells were co-cultured with resting NK cells for 72 hours. **C**, NK cell-mediated specific lysis of iT cells, calculated as $(1 - \text{signal in NK cell group} / \text{signal in control group}) \times 100\%$, assessed at 72 hours. P values were determined using a two-tailed, unpaired t-test. Data are mean $\pm$ s.d. (n = 7 per group). **D**, Experimental design of the immunogenicity assay using IL-15-pretreated NK cells. Parental iT cells were co-cultured for 48 hours. **E**, Specific lysis of parental iT cells in the absence or presence of IL-15-treated NK cells.



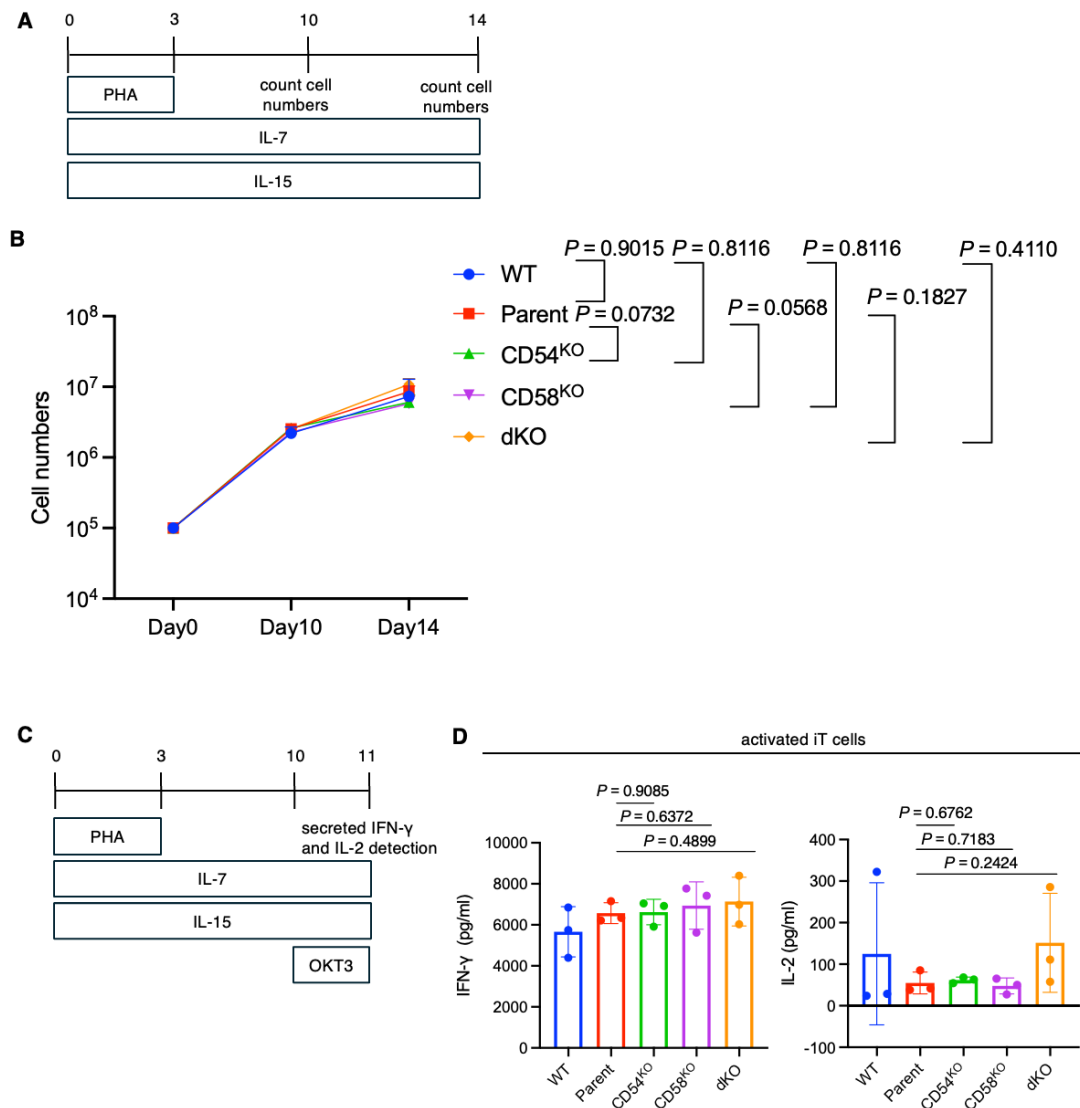
Supplementary Figure 2 | Expression of NK cell surface antigens targeted by HLA-E and PVR.

A, Flow cytometry analysis of CD159a, DNAM-1 and TiGiT expression on NK cells from three independent donors.



Supplementary Figure 3 | Gating strategy.

A, Gating strategy for identifying CD4⁺CD8⁺ cells. **B**, Gating strategy for identifying CD8αβ⁺ cells. **C**, Nalm6 cells were gated based on GFP⁺ hKO-1⁻ expression after coculture with iCAR-T cells. **D**, Gating GFP⁻ as NK cells after coculture with iT cells. CD107a expression was measured within the gated NK cell population.



Supplementary Figure 4 | Effector function of iT cells.

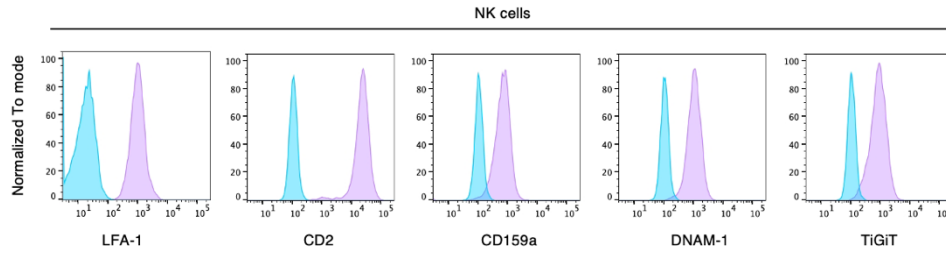
A, Experimental timeline. iT cells were activated with PHA-stimulated PBMCs for 3 days. Cell numbers were assessed on Days 10 and 14.

B, Cell numbers of WT, parental iT, CD54^{KO} iT, CD58^{KO} iT, and dKO iT cells on Days 0, 10, and 14. Statistical significance was assessed by two-way ANOVA with multiple comparisons ($n = 3$ per group).

C, Experimental timeline. iT cells were stimulated with OKT3 on Day 10 following PHA/PBMCs expansion. Cytokine production was analyzed 24 hours after OKT3 stimulation.

D, Expression levels of IFN-γ and IL-2 in activated iT cells. Statistical analysis was performed using a two-tailed unpaired t-test. Data are presented as mean $\pm$ s.d. ($n = 3$ per group).

A



Supplementary Figure 5 | Surface expression of NK cell receptors in donor #4.

A, Flow cytometry analysis of LFA-1, CD2, CD159a, DNAM-1 and TiGiT expression on NK cells from donor4.

Antibody	Source	Identifier
CD4 BV421	BioLegend	317434; RRID: AB_2562134
CD8b PE-Cy7	eBioscience	25-5273-42; RRID: AB_11219680
CD34 PB	BioLegend	343512; RRID: AB_1877197
CD43 FITC	BD	555475; RRID: AB_395867
CD56 APC-Cy7	BioLegend	318332; RRID: AB_10896424
CD107a APC	BioLegend	328620; RRID:AB_1279055
CD107a BV421	BioLegend	328626; RRID:AB_11203537
HLA-ABC APC	BioLegend	311410; RRID:AB_314879
CD54 APC	BioLegend	353112; RRID:AB_10916103
CD58 PE	BioLegend	330905; RRID:AB_1186063

Supplementary Table 1 | Key Resources

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