

A Robust Protocol for Differentiation and Expansion of hPSC-derived NK Cells

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Abstract

Natural killer (NK) cells are critical innate lymphocytes that serve as the first line of defense against tumor and virus-infected cells by exerting major histocompatibility complex (MHC)-unrestricted cytotoxicity and regulating adaptive immunity through cytokine secretion. However, the clinical application of primary NK (pNK) cells is limited by low yield, inefficient gene transduction, and donor-to-donor variability. Human pluripotent stem cell (hPSC)-induced NK (iNK) cells represent a promising "off-the-shelf" cell source for cancer immunotherapy, enabling the scalable production of homogeneous iNK cells. Here, we present a standardized and reproducible protocol for the directed differentiation of hPSCs into functional iNK cells, along with their efficient ex vivo expansion. We define critical procedural steps, optimized culture conditions, key parameters, and quality control criteria to ensure consistency and functionality. This protocol offers a valuable resource for preclinical research and facilitates the development of standardized, off-the-shelf NK cell therapies for oncology applications.

Keywords

Human pluripotent stem cell, induced NK cell, Differentiation, Cell therapy

Introduction

Natural killer (NK) cells are pivotal innate immune lymphocytes that mediate rapid cytotoxic responses against transformed and virus-infected cells without prior antigen sensitization and modulate adaptive immunity through the secretion of cytokines and chemokines (Lanier 2024). Importantly, the strength of NK cell activity is closely associated with the incidence and progression of cancers and other diseases (Wu *et al.* 2025; Imai *et al.* 2000). These unique properties make NK cells highly attractive for adoptive cellular immunotherapy in both hematological malignancies and solid tumors (Vivier *et al.* 2024). However, the clinical application of pNK cells derived from peripheral blood (PB) or umbilical cord blood (UCB) has been constrained by several limitations, including limited cell source, donor-to-donor functional variability leading to inconsistent product performance, and relatively low efficiency of gene editing/engineering, all of which hinder their broad clinical application.

Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), offer an attractive alternative source for NK cell generation owing to their unlimited self-renewal capacity and multilineage differentiation potential (Wei *et al.* 2025; Zhao *et al.* 2023; Gheitsi *et al.* 2024). iNK cells enable the scalable production of homogeneous cell populations with consistent phenotypic and functional properties (Woll *et al.* 2009). In addition, hPSC platforms facilitate precise genetic engineering, allowing the development of engineered NK cells with enhanced antitumor activity (Kumar *et al.* 2025; Li *et al.* 2018). Importantly, iNK cells exhibit favorable safety profiles, with negligible cytokine release syndrome (CRS), neurotoxicity, and graft-versus-host disease (GvHD), thereby positioning them as next-generation standardized “off-the-shelf” cellular therapeutics (Wang *et al.* 2025).

To date, multiple differentiation strategies have been developed to generate NK cells from hPSCs, yet many suffer from long timelines, reliance on feeder cells or complex cytokine cocktails, low purity, and poor reproducibility. For example, early protocols based on xenogeneic stromal feeder cells such as OP9 to obtain mature iNK cells and introduced undesired xenogeneic impurities and biosafety hazards, hindering their further clinical development (Huang *et al.* 2022). Embryoid body (EB)-based approaches, while widely adopted, also demand extended culture periods of 40 days or longer and generate

heterogeneous cell populations that often require fluorescence-activated cell sorting (FACS) or magnetic enrichment to achieve sufficiently pure CD56⁺ NK cells (Qin *et al.* 2024). Even newer feeder-free systems frequently rely on complicated cytokine combinations and still exhibit low final purity and inconsistent yields (Lupo *et al.* 2021). These challenges underscore the need for a simplified, robust, and standardized protocol that enables efficient and scalable production of functional iNK cells.

Here, we present a feeder-free, step-by-step protocol for the generation of iNK cells from hiPSCs. This protocol simplifies cultural workflows, substantially shortens the entire differentiation timeline, and reduces experimental costs, while remarkably elevates the purity of terminal NK cells without the requirement of additional cell sorting. Specifically, hiPSCs are directionally differentiated into hematopoietic and lymphoid progenitors, which are further matured and expanded to generate functional iNK cells. Flow cytometry verified that the iNK cells harvested from this system exert potent cytotoxic activity and retain minimal residual pluripotent cells. Additionally, we provide detailed guidance on reagent preparation, critical operational steps, quality control markers, and troubleshooting strategies to improve differentiation efficiency and ensure reproducibility. Collectively, this streamlined and robust platform enables the high-yield and high-purity production of iNK cells, which provides a reliable tool for mechanistic research and promotes the clinical translation of iNK-based immunotherapies.

Summarized Procedure

The protocol consists of three sections. The first section provides a detailed procedure for differentiating human pluripotent stem cells into iNK cells. Importantly, assessment of pluripotency prior to differentiation is a prerequisite for achieving optimal outcomes. Pluripotency is evaluated by flow cytometric analysis of key markers, including SSEA-4, TRA-1-60, SOX2, and NANOG, and only cell populations with >95% positivity are used for subsequent differentiation. In this study, we established a standardized three-stage protocol for the generation of iNK cells from hPSCs. Briefly, hPSCs were first induced to differentiate into CD34⁺ hematopoietic stem/progenitor cells (HSPCs), followed by further specification into common lymphoid progenitors (CLPs) characterized as CD5⁻CD7⁺CD45⁺ cells. These

CLPs were subsequently directed to differentiate into mature iNK cells exhibiting a typical immunophenotype (CD3⁻CD45⁺CD56⁺CD16⁺). Flow cytometric analysis of pluripotency markers confirmed the absence of residual pluripotent stem cells in the final iNK product, ensuring high safety.

In the second section, we provide detailed guidelines for the ex vivo expansion of iNK cells. The cells undergo one to three rounds of expansion, resulting in a several hundred- to thousand-fold increase in cell number, accompanied by enhanced expression of activation-associated markers such as CD16. Notably, the proportion of CD56⁺ cells reaches up to 95% after expansion, indicating a highly purified NK cell population.

In the third section, we describe the functional characterization of iNK cells in detail. Expanded iNK cells exhibit high expression of activating receptors (NKp46, NKG2D). For exhaustion-associated receptors, TIM-3 displays relatively high expression, while LAG-3, PD-1, as well as inhibitory receptors (NKG2A, TIGIT), are expressed at low levels. Upon co-culture with A549 tumor cells, iNK cells exhibit robust cytokine secretion profiles. Compared with peripheral blood NK (PBNK) cells, these iNK cells exert superior cytotoxic activity against A549 cells in vitro, demonstrating promising potential for cancer immunotherapy applications. A schematic workflow of the protocol is shown in Fig. 1.

MATERIALS AND EQUIPMENT

MATERIALS

Reagents

- STEMdiff™ Hematopoietic - EB Basal Medium (STEMCELL Technologies, Cat. #100-0171)
- STEMdiff™ Hematopoietic - EB Supplement A (STEMCELL Technologies, Cat. #100-0172)
- STEMdiff™ Hematopoietic - EB Supplement B (STEMCELL Technologies, Cat. #100-0173)
- StemSpan™ Lymphoid Progenitor Expansion Supplement (10X) (STEMCELL Technologies, Cat. #09915)
- StemSpan™ Lymphoid Differentiation Coating Material (100X) (STEMCELL Technologies, Cat. #09925)
- StemSpan™ NK Cell Differentiation Supplement (10X) (STEMCELL Technologies, Cat. #09950)
- StemSpan™ SFEM II (STEMCELL Technologies, Cat. #09605)

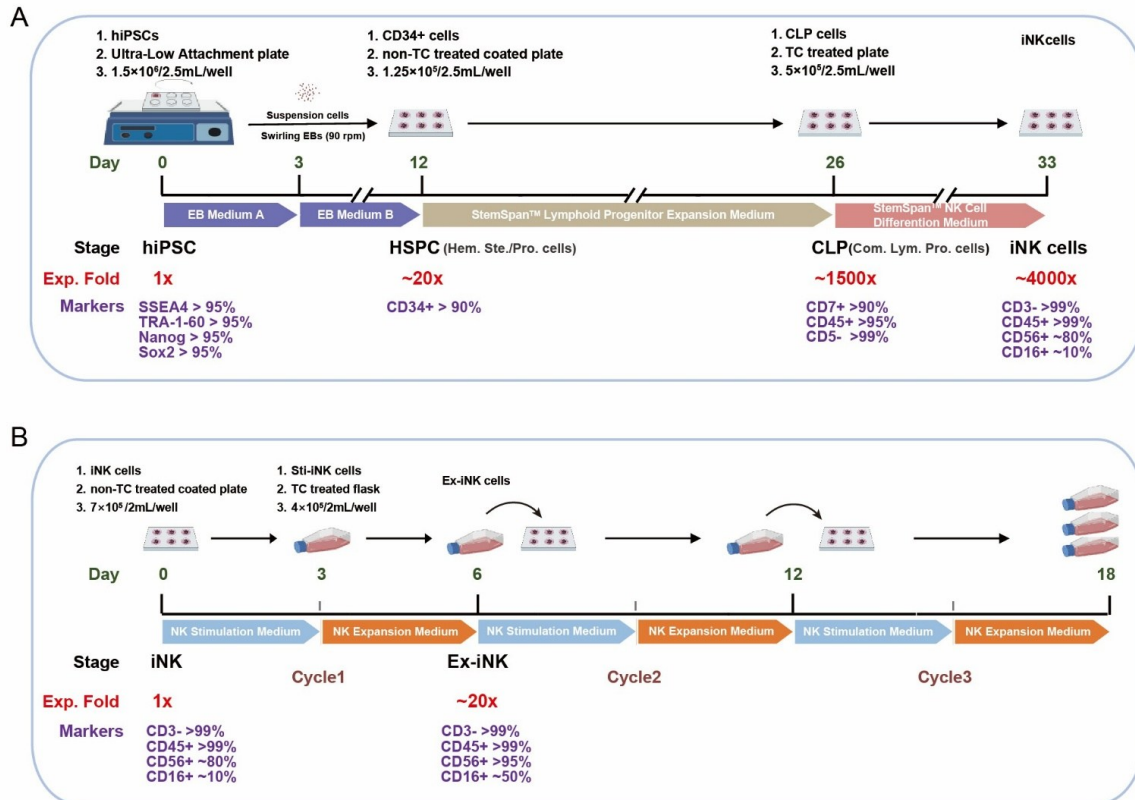


Fig. 1 Workflow for hiPSC differentiation into iNK cells and ex vivo expansion. A. Schematic diagram showing the differentiation of hiPSCs into iNK cells. **B.** Schematic diagram showing the expansion of differentiated iNK cells.

- mTeSR™ Plus (STEMCELL Technologies, Cat. #100-0274)
- UM729 (STEMCELL Technologies, Cat. #72332)
- CryoStor® CS10 (STEMCELL Technologies, Cat. #100-1061)
- Accutase (STEMCELL Technologies, Cat. #7922)
- DPBS (Gibco, Cat. #14190250)
- EDTA (Invitrogen, Cat. #AM9261)
- TrypLE™ Express Enzyme (Gibco, Cat. #12605028)
- Y-27632 (MedChemExpress, Cat. #HY-10583)
- VTN-N (Gibco, Cat. #A31804)
- NK Xpander (Gibco, Cat. #A5019001)
- RPMI 1640 Medium (Thermo Fisher Scientific, Cat. #11875119)
- Recombinant Human IL-15 Protein (Sino Biological, Cat. #10360-HNCE)
- Recombinant Human IL-21 Protein (Sino Biological, Cat. #10584-HNAE)
- Recombinant Human IL-18 Protein (Sino Biological, Cat. #10119-HNCE)
- Human AB Serum (Heat Inactivated) (valley biomedical, Cat. #HP1022HI)
- TRYPAN BLUE STAIN (Invitrogen, Cat. #15250061)

Antibodies

- PE Mouse Anti-Human CD34 (BD Pharmingen, Cat. #550761, Clone. 563)
- APC Mouse Anti-Human CD7 (BD Pharmingen, Cat. #561604, Clone. M-T701)
- FITC Mouse Anti-Human CD5 (BD Pharmingen, Cat. #561896, Clone. UCHT2)
- BV421 Mouse Anti-Human CD45 (BD Pharmingen, Cat. #563879, Clone. HI30)
- PE Mouse Anti-Human CD56 (BD Pharmingen, Cat. #563238, Clone. R19-760)
- APC Mouse Anti-Human CD16 (BD Pharmingen, Cat. #561248, Clone. 3G8)
- FITC-CD3 (BD Pharmingen, Cat. #555339, Clone. HIT3 α)
- APC anti-human CD314 (NKG2D) (BioLegend, Cat. #320807, Clone. 1D11)
- APC anti-human CD335 (NKp46) (BioLegend, Cat. #331918, Clone. 9E2)
- APC/Cyanine7 anti-human TIGIT (BioLegend, Cat. #372734, Clone.A15153G)
- BV421 Mouse Anti-Human LAG-3 (CD223) (BD Pharmingen, Cat. #565721, Clone. T47-530)
- PE Mouse Anti-Human TIM-3 (CD366) (BD Pharmingen, Cat. #565570, Clone. 7D3)
- FITC Mouse Anti-Human CD279 (BD Pharmingen, Cat. #561035, Clone. MIH4)
- BD Horizon™ BV421 Mouse Anti-Human TRA-1-60 (BD Pharmingen, Cat. #562711, Clone. TRA-1-60)
- Alexa Fluor® 488 Mouse Anti-Human Nanog (BD Pharmingen, Cat. #560791, Clone. N31-355)
- Alexa Fluor® 647 Mouse Anti-SSEA-4 (BD Pharmingen, Cat. #560796, Clone. MC813-70)
- PE Mouse Anti-Sox2 (BD Pharmingen, Cat. #562195, Clone. O30-678)
- Human NKp46/NCR1 Antibody (R&D Systems, Cat. #MAB1850-SP, Clone. 195314)
- CD16 Monoclonal Antibody (Thermo Fisher Scientific, Cat. #16-0166-85, Clone. 3G8)

Human cell sources

- Commercial human-induced pluripotent stem cells (hiPSCs, UniXell Biotechnology, Cat. #iPSC-YS006-30, Bat. #0520250606)
- Commercial peripheral-blood-derived NK cells (PBNK, Shenzhen Cell Valley, Cat. #SZCV2026-CV103, Bat. #GZSYS20260602)

Equipment

- Centrifuge (Thermo Fisher Scientific, MicroCL 21R)
- Flow cytometer (BD, FACS Aria III)
- Cell incubator, 5% CO₂, 21% O₂ (Thermo Fisher, 150i)
- Orbital shaker
- Class II biological safety cabinet
- Automated cell counter
- Ultra-low attachment 6-well plate (Corning, Cat. #07200-601)
- Non-treated 6-well plate (nTC 6WP)
- Tissue-culture treated 6-well plate (TC 6WP)

Reagent Setup

Table 1 VTN-N Coating Solution (per 6-well plate)

Composition	Volume	Final concentration
VTN-N (0.5 mg/mL)	120 µL	6.6 µg/mL
DPBS (Ca ²⁺ /Mg ²⁺ -free)	9 mL	—
Total	9.12 mL	1 µg/cm ² per well

Table 2 EB Formation Medium (per 5 mL)

Composition	Volume	Final concentration
EB Medium A	5 mL	—
Y-27632 (10 mM)	5 µL	10 µM

Table 3 EB Medium A (per 7.5 mL)

Composition	Volume
STEMdiff™ Hematopoietic - EB Basal Medium	7.5 mL
EB Supplement A	37.5 µL

Table 4 EB Medium B (per 10 mL)

Composition	Volume
STEMdiff™ Hematopoietic - EB Basal Medium	9 mL
EB Supplement B	1 mL

Table 5 Lymphoid Progenitor Expansion Medium (per 10 mL)

Composition	Volume
StemSpan™ SFEM II	9 mL
Lymphoid Progenitor Expansion Supplement (10X)	1 mL

Table 6 NK Cell Differentiation Medium (per 10 mL)

Composition	Volume	Final concentration
StemSpan™ SFEM II	9.9 mL	—
NK Cell Differentiation Supplement (100X)	100 µL	—
UM729 (2.5 mM)	4 µL	1 µM

Table 7 NK Cell Expansion - Stimulation Medium (per 2 mL)

Composition	Volume	Final concentration
NK Xpander	1.8 mL	—
Human AB Serum	0.2 mL	10% (v/v)
IL-15 (250ug/mL)	0.08 µL	10 ng/mL
IL-18 (250ug/mL)	0.08 µL	10 ng/mL
IL-21 (250ug/mL)	0.08 µL	10 ng/mL

Table 8 NK Cell Expansion Medium (per 2 mL)

Composition	Volume	Final concentration
NK Xpander	1.8 mL	—
Human AB Serum	0.2 mL	10% (v/v)
IL-15 (250ug/mL)	0.08 µL	10 ng/mL
IL-18 (250ug/mL)	0.08 µL	10 ng/mL

PROCEDURE

Section 1 Differentiation of human pluripotent stem cells into iNK cells

Stage 1 – Differentiation of hPSCs into CD34⁺ HSPCs [TIMING ~12 days]

Step 1.1: Maintain hPSCs on VTN-coated plates in mTeSR medium (Fig. 2A). Three to four days after seeding, pluripotency validation is performed by analyzing pluripotency markers via flow cytometry, including the surface markers TRA-1-60 and SSEA-4 as well as the nuclear markers Sox2 and Nanog. Only hPSCs with $\geq 95\%$ positivity for these pluripotency markers are used for differentiation (Fig. 2B).

Step 1.2: Rinse hPSCs with DPBS. Add Accutase and incubate at 37 °C for 5 min.

Step 1.3: Add culture medium to neutralize Accutase and gently pipette to obtain a single-cell suspension. Determine viable cell number.

Step 1.4: Centrifuge cells at 1,250 rpm for 5 min. Resuspend cells in 2.5 mL EB Formation Medium at a final concentration of 6×10^5 cells/mL and seed into ultra-low attachment six-well plates.

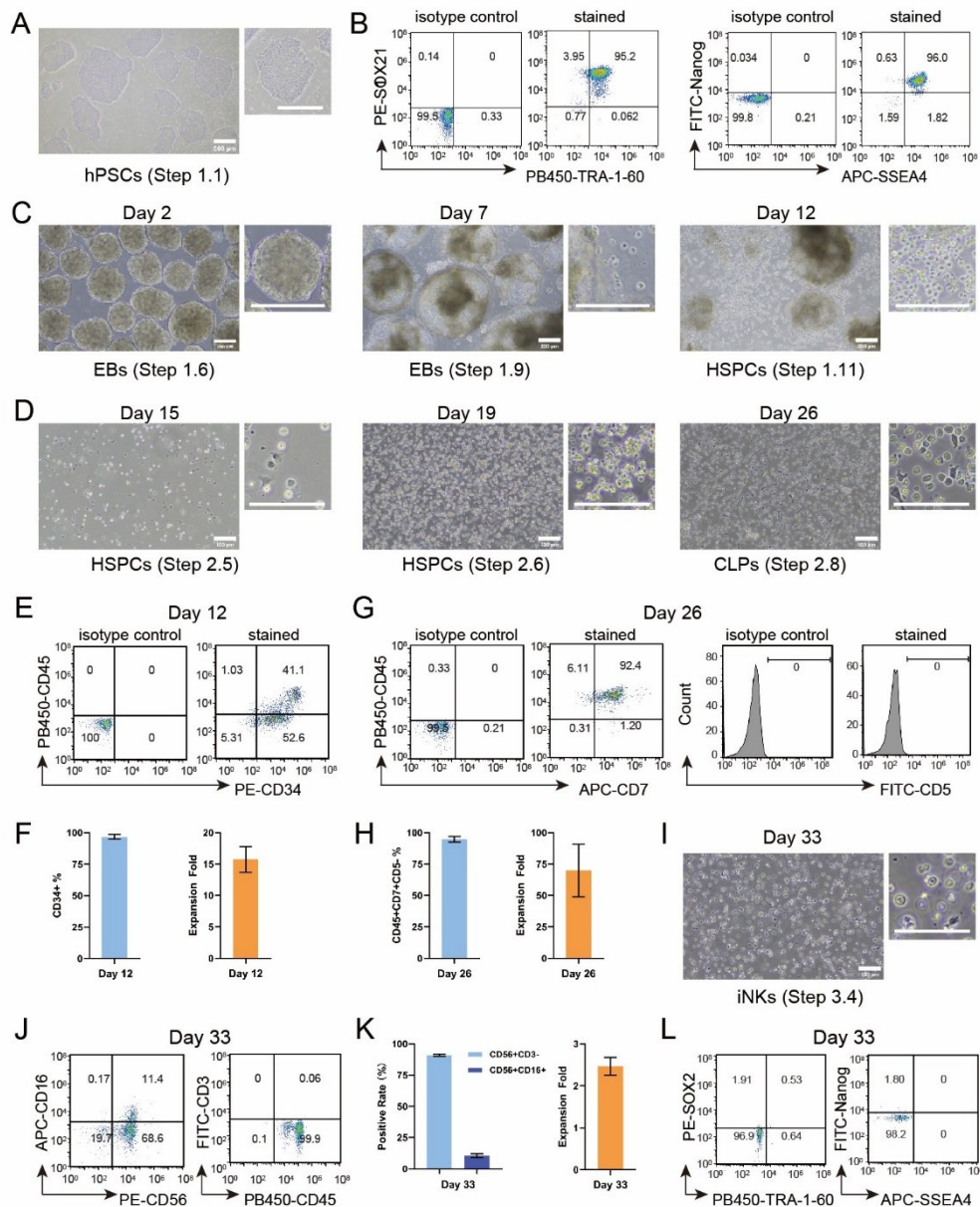


Fig. 2 Differentiation of hiPSC into iNKs. **A.** Representative micrograph of hiPSCs with favorable growth status. Scale bars, 500 μ m. **B.** Flow cytometric analysis of pluripotency markers SSEA4, TRA-1-60, SOX2, and Nanog in hiPSCs. **C.** Representative morphological images of embryoid bodies (EBs) at day 2 of differentiation (left), EBs at day 7 of differentiation (middle), and hematopoietic stem/progenitor cells (HSPCs) released from EBs after 12 days of differentiation (right). Scale bars, 200 μ m. **D.** Representative morphological images showing the transition from HSPCs to common lymphoid progenitors (CLPs). Scale bars, 100 μ m. **E.** Representative flow cytometric analysis of HSPCs at day 12 of differentiation. **F.** Bar graphs show the percentage of CD34⁺ cells (blue) and expansion fold (orange) across three independent differentiation batches (n=3, mean $\pm$ SD). **G.** Representative flow cytometric analysis of CLPs at day 26 of differentiation. **H.** Bar graphs show the percentage of CD45⁺CD7⁺CD5⁻ cells (blue) and expansion fold from differentiation day 12 to day 26 (orange) across three independent differentiation batches (n=3, mean

±SD). **I.** Representative morphological image of iNK cells at day 33 of differentiation. Scale bars, 100 µm. **J.** Representative flow cytometric analysis of iNK cells at day 33 of differentiation. **K.** Bar graphs show the percentages of CD56⁺CD3⁻ and CD56⁺CD16⁺ cells (blue), as well as expansion fold from differentiation day 26 to day 33 (orange), across three independent differentiation batches (n=3, mean±SD). **L.** Flow cytometric detection of residual pluripotent stem cells in iNK cells on day 33 of differentiation.

Step 1.5: Place the plate on an orbital shaker at 90 rpm and incubate at 37 °C with 5% CO₂ (designated as Day 0).

[CRITICAL STEP] hPSCs must be dissociated into a high-quality single-cell suspension using minimal mechanical stress to preserve viability. Optimal seeding density is critical for efficient hPSCs secretion and may vary among hPSC lines. For our hiPSC line, 1.5×10^6 cells /well produced optimal results.

Step 1.6: On Day 2, remove 2.5 mL of medium and add 2.5 mL fresh EB Medium A (Fig. 2C).

Step 1.7: On the next day, remove 2.5 mL of medium and add 3 mL EB Medium B. Incubate for an additional 2 days.

Step 1.8: On Day 5, remove 2.5 mL of medium and centrifuge collected suspension at $120 \times g$ for 10 min. Discard the supernatant. Resuspend the pellet in 3 mL EB Medium B and return to the original well.

[CRITICAL STEP] Beginning on Day 5, increasing numbers of cells are released from EB spheres into the culture supernatant. During medium changes, collect the supernatant and retrieve detached cells by low-speed centrifugation to maximize cell viability and recovery.

Step 1.9: On Day 7, remove 3.5 mL of medium and centrifuge at $120 \times g$ for 10 min. Resuspend the cells in 4 mL EB Medium B and return them to the original well (Fig. 2C).

[CRITICAL STEP] From now on, the culture medium becomes conspicuously yellow, and a large number of secreted cells accumulate in the culture supernatant. Timely medium replacement is essential to maintain cell viability.

Step 1.10: On Day 10, remove 4 mL of medium and collect the cells by centrifugation. Resuspend the cells in 4.5 mL EB Medium B and return them to the original well.

Step 1.11: On Day 12, collect suspension cells released from EBs for cell counting, and assess CD34 expression by flow cytometry (Fig. 2C, 2E, and 2F). The proportion of CD34⁺ cells typically exceeds 90%, allowing direct transition to **Stage 2** without cell sorting.

Stage 2 – Differentiation into CLPs [TIMING ~14 days]

Step 2.1: Coat non-tissue culture-treated (nTC) six-well plates with Lymphoid Differentiation Coating Material and incubate for 2 h at room temperature or overnight at 4 °C.

Step 2.2: Collect suspension cells and gently rinse once with PBS. Centrifuge at 120×g for 10 min.

Step 2.3: Resuspend CD34⁺ cells in 2.5 mL Lymphoid Progenitor Expansion Medium and seed at 5×10^4 cells/mL. Calculate the absolute number of CD34⁺ cells according to the following formula: CD34⁺ cell number = viable cell number × flow cytometry percentage of CD34⁺ cell.

Step 2.4: Incubate cultures at 37 °C with 5% CO₂ for 72 h.

Step 2.5: On Day 15, add 1.5 mL fresh medium to each well and continue incubation for 96 h (Fig. 2D). At this point, the viable cell count had increased approximately 1- to 3-fold relative to the initial CD34⁺ cells seeding.

Step 2.6: On Day 19, transfer cells at a 1:1 ratio into newly coated nTC six-well plate. First, collect 2 mL of supernatant from the edge of each well and centrifuge at 120×g for 10 min. During centrifugation, rinse the pre-coated nTC six-well plate once with PBS. Gently resuspend the remaining cells in the original wells by pipetting and transfer the cell suspension into the new plates. Rinse each original well with 1 mL fresh Lymphoid Progenitor Expansion Medium to recover residual cells and add the rinse to the corresponding new well. After centrifugation, resuspend the cell pellet in 1 mL Lymphoid Progenitor Expansion Medium and transfer it to the corresponding well. The final culture volume in each well should be approximately 4 mL (Fig. 2D). At this point, the viable cell count had increased approximately 20- to 30-fold relative to the initial CD34⁺ cells seeding (Fig. 2D). Continue incubation for 72 h.

Step 2.7: On Day 22, remove 1.5 mL medium and collect the cells by centrifugation. Resuspend and return the cells to the original well. Continue incubation for 96 h.

Step 2.8: On Day 26, cells were harvested for cell counting, and the CLP phenotype (CD5⁻CD7⁺CD45⁺) was assessed via flow cytometry (Fig. 2G and 2H). At this point, the viable cell count had increased approximately 55- to 95-fold relative to the starting CD34⁺ cell number.

Stage 3 – Differentiation into mature iNK Cells [TIMING ~7 days]

Step 3.1: Resuspend CLPs in 2.5 mL NK Cell Differentiation Medium and seed into tissue culture-treated six-well plates at 2.0×10^5 cells/mL.

Step 3.2: Incubate at 37 °C with 5% CO₂ for 72 h.

Step 3.3: On Day 29, add 2.5 mL fresh NK Cell Differentiation Medium and continue incubation for 96 h. At this point, the cell number showed minimal change or only a slight increase (up to ~1.5-fold).

Step 3.4: On Day 33, collect differentiated cells. At this point, the viable cell count had increased approximately 2- to 3-fold relative to the initial CLPs seeding. Analyze the mature NK cell phenotype (CD45⁺CD3⁻CD56⁺CD16⁺) (Fig. 2I, 2J, and 2K) and validate absence of residual hPSCs (Nanog, SSEA4, TRA-1-60, Sox2 negative) by flow cytometry (Fig. 2L).

Section 2 Expansion of iNK cells [TIMING ~6 days per cycle]

Stage 4: Stimulation Phase (Day 0–3)

Step 4.1: Coat nTC six-well plates with anti-CD16 antibody (5 µg/mL), anti-NKp46 antibody (5 µg/mL), and RetroNectin (20 µg/mL) using 1.5 mL coating solution per well. Incubate overnight at 4 °C or for 2 h at room temperature.

Step 4.2: Collect mature iNK cells and centrifuge at 120×g for 10 min. Resuspend cells in Stimulation Medium at a density of 3.5×10^5 cells/mL. Before seeding the cells, remove the coating solution from the pre-coated wells and rinse each well once with D-PBS. Add 2 mL of the cell suspension to each well and incubate at 37 °C with 5% CO₂ for 72 h (Fig. 3A).

Stage 5: Expansion Phase (Day 3–6)

Step 5.1: Collect stimulated iNK cells (Sti-iNKs) and centrifuge at 120×g for 10 min. Resuspend the cells in Expansion Medium at a final density of 2×10^5 cells/mL and transfer 15 mL of cell suspension into a T75 flask. Incubate at 37 °C with 5% CO₂ for 72 h (Fig. 3A).

Step 5.2: Collect cells after one round of expansion, and determine cell viability via Trypan blue staining ($95\% \pm 3\%$). Assess Ex-iNKs purity and phenotype by flow cytometry, including the expression of CD56 and CD16 (Fig. 3B and 3C).

Repeat stimulation–expansion cycles as needed for large-scale iNK cell manufacturing.

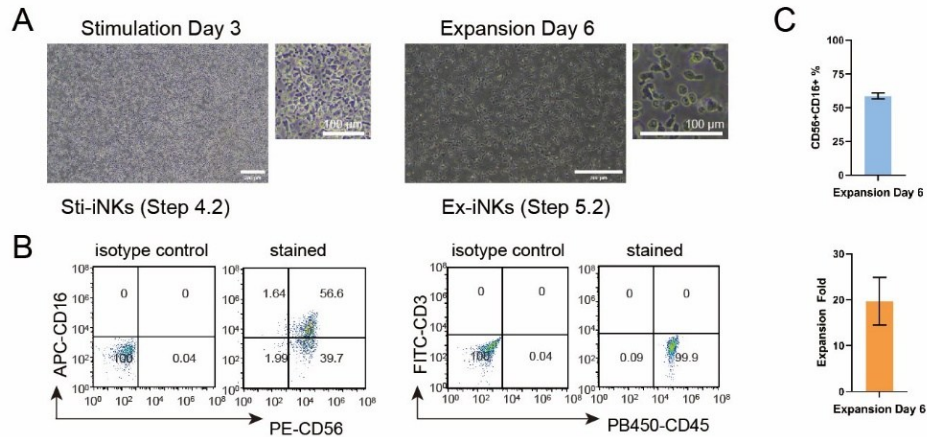


Fig. 3 Expansion of iNK cells. **A.** Representative micrographs of iNK cells with favorable growth status on day 3 (left, after 3 days of stimulation) and day 6 of expansion (right, after 3 days of expansion). Scale bars, 200 μm ; magnified images, 100 μm . **B.** Representative flow cytometric analysis of expanded iNK cells (Ex-iNKs) at day 6 following one round of expansion. **C.** Bar graphs showing the percentage of CD56⁺CD16⁺ cells (blue) and expansion fold for one round of expansion (orange) from three independent expansion batches (n=3, mean $\pm$ SD).

Section 3 Functional characterization of iNK cells

1. Analysis of functional receptors:

(A) Collect expanded iNK cells (Ex-iNKs) and centrifuge at $120\times g$ for 10 min.

(B) Resuspend Ex-iNK or PBNK cells in RPMI 1640 medium and adjust the cell density to obtain effector-to-target (E:T) ratios of 2:1, 5:1, and 8:1. Add 100 μL of Ex-iNK or PBNK cell suspension to each experimental well, while 100 μL RPMI 1640 medium is added to control wells containing tumor cells alone. Three technical replicates were prepared for each condition. The plates were placed in the Incucyte S3 live-cell imaging system, and images

were acquired every 30 min over a 24-h period to assess tumor-cell killing activity (Fig. 4D-4G).

2. Cytotoxicity function assessment:

(A) Seed A549 cells into 96-well plates one day before co-culture at 9,000 cells per well in 100 μ L RPMI 1640 medium.

(B) Activating and inhibitory receptors (NKG2D, NKp46, TIGIT, and NKG2A), exhaustion-associated markers (TIM-3, PD-1, and LAG-3) (Fig. 4A and 4C), as well as cytokine secretion markers (TNF- α , GZMB, and IFN- γ) after co-culture with A549 tumor cells (Fig. 4B), were detected by flow cytometry.

TROUBLESHOOTING

Troubleshooting suggestion can be find in Table 9.

Table 9 Troubleshooting table

Description	Possible reason	Suggestion
Low CD34 ⁺ HSPC yield at Stage 1	Poor hPSC pluripotency; inappropriate EB size or density; medium acidification caused by delayed medium replacement	Use hPSCs with $\geq 95\%$ positivity for TRA-1-60, SSEA-4, SOX2, and NANOG. Optimize EB seeding density and perform timely medium changes, particularly after Day 7 when rapid proliferation increases medium acidification.
Low iNK differentiation efficiency (low CD56 ⁺ CD16 ⁺)	Poor CLP quality	Confirm high CD7 positivity during the CLP stage (preferably $>90\%$).
Poor iNK expansion	Incomplete antibody or RetroNectin coating; inappropriate cell density	Prepare coating solution carefully to ensure complete plate coverage. Maintain recommended stimulation and expansion densities throughout culture.

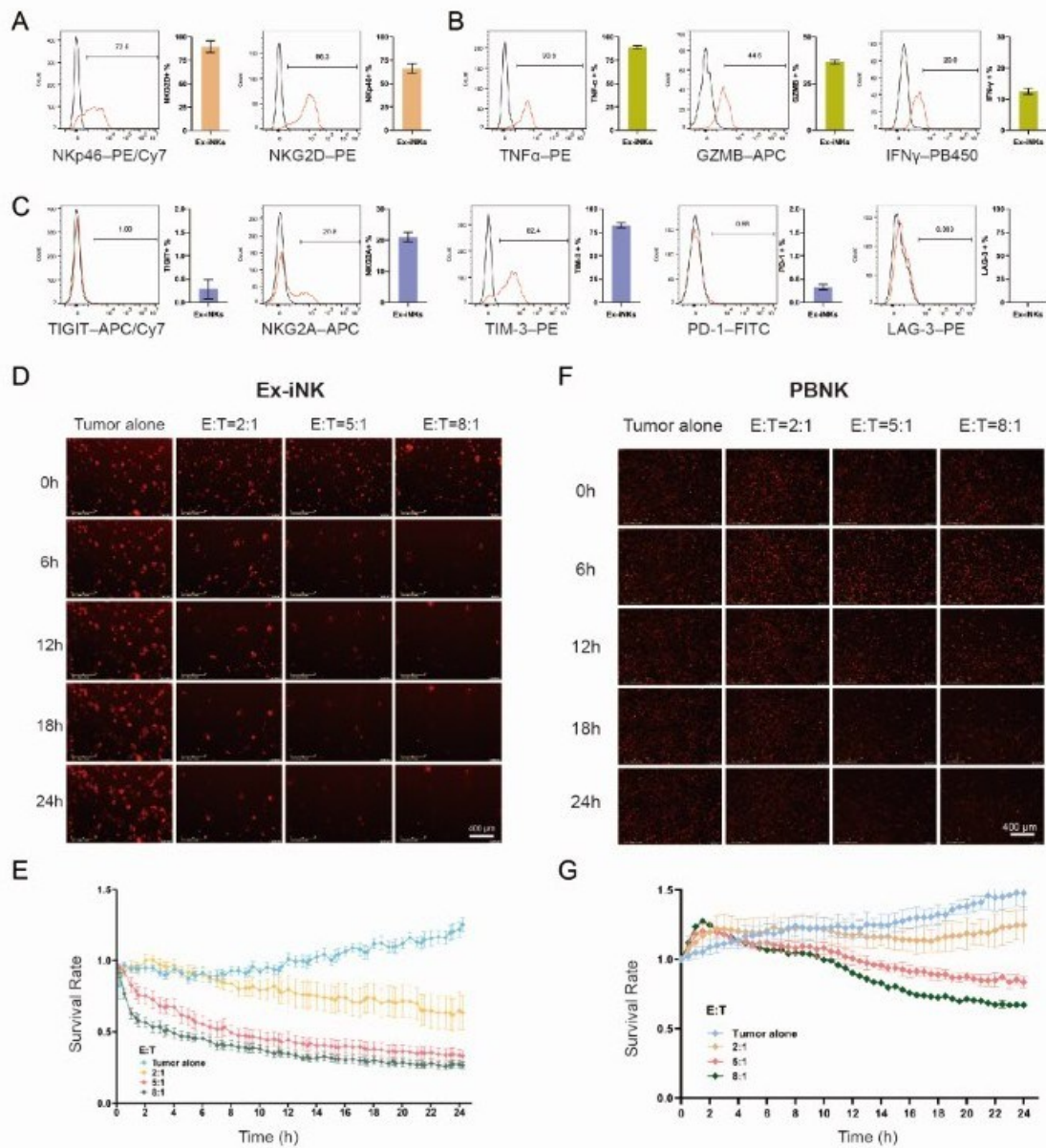


Fig. 4 Functional validation of Ex-iNKs. **A.** Representative flow cytometric analysis of activating receptor (NKp46, NKG2D) expression on expanded iNK cells (Ex-iNKs). Bar graphs show the relative expression levels of these indicators across three independent batches (n=3, mean ± SD). **B.** Representative flow cytometric analysis of cytokine secretion (TNF-α, GZMB, IFN-γ) in Ex-iNKs. Bar graphs show the relative expression levels of these indicators across three independent batches (n=3, mean ± SD). **C.** Representative flow cytometric analysis of inhibitory receptors (TIGIT, NKG2A) and exhaustion-associated markers (TIM-3, PD-1, LAG-3) on Ex-iNKs. Bar graphs show the relative expression levels of these indicators across three independent batches (n=3, mean ± SD). **D.** Representative micrographs of Ex-iNKs co-cultured with A549 tumor cells at E:T ratios of 2:1, 5:1, and 8:1 at 0, 6, 12, 18, and 24 h, along with mono-cultured A549 tumor cells as controls. Scale bars, 400 μm. **E.** Real-time cell analysis (RTCA)-based killing assays were performed to evaluate the cytotoxic activity of Ex-iNKs against A549 cells (n=3, mean ± SD). **F.** Representative micrographs of PBNKs co-cultured with A549 tumor cells

at E:T ratios of 2:1, 5:1, and 8:1 at 0, 6, 12, 18, and 24 h, along with mono-cultured A549 tumor cells as controls. Scale bars, 400 μ m. **G.** Real-time cell analysis (RTCA)-based killing assays were performed to evaluate the cytotoxic activity of PBNKs against A549 cells (n=3, mean $\pm$ SD).

ADVANTAGES AND LIMITATIONS OF THE PROTOCOL

This protocol provides a robust and scalable strategy for generating iNK cells from human pluripotent stem cells. The differentiation process is performed under feeder-free and serum-free conditions, thereby improving experimental reproducibility and facilitating future clinical translation. Another major advantage of this protocol is without the need for cell sorting, reducing cell loss and simplifying the workflow. In addition, the overall differentiation timeline is relatively short, requiring approximately 33 days to generate mature iNK cells from hPSCs, while each stimulation – expansion cycle requires only 6 days, enabling the rapid and scalable production of NK cells.

Importantly, the present protocol should be considered an optimized and simplified adaptation of established NK cell differentiation and expansion platforms rather than a fundamentally new differentiation system. The iNK differentiation process is based on the commercially available STEMdiff™ NK Cell Differentiation platform, with systematic optimization of culture configuration, medium-exchange strategy, sorting requirements, and differentiation timeline. The expansion phase is based on the scalable NK cell expansion strategy reported by Kikuchi *et al.* (2025) (Kikuchi, Takeuchi, and Yamaguchi 2025). The system-specific advantages of the present protocol therefore primarily lie in its sorting-free workflow, simplified culture operation, shortened differentiation period, and practical scalability.

Several limitations remain. First, the differentiation process currently relies heavily on specialized commercial media and reagents from STEMCELL Technologies, which may increase manufacturing costs and limit flexibility for process optimization. Second, the current expansion strategy is based primarily on static culture systems, which may restrict large-scale NK cell production and nutrient exchange efficiency during prolonged culture. The future development of wave bioreactor systems or stirred-suspension culture platforms

may further improve NK cell expansion efficiency, scalability, and manufacturing consistency (Kikuchi, Takeuchi, and Yamaguchi 2025). Third, cryopreservation is an important consideration for realizing the “off-the-shelf” potential of iNK cells. In the present protocol, we performed preliminary cryopreservation experiments and found that Ex-iNK cells maintained >85% viability after thawing with satisfactory cell recovery. However, post-thaw functional recovery was not systematically evaluated. In particular, comprehensive assessment of cytotoxicity, cytokine production, and other effector functions after cryopreservation will be important for fully establishing the “off-the-shelf” potential of these iNK cells.

Collectively, this protocol provides a practical and reproducible platform for generating functional iNK cells from hPSCs and offers broad utility for studies of NK cell biology, cancer immunotherapy, genetic engineering, and scalable cell manufacturing.

Acknowledgements

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Compliance with Ethical Standards

Conflict of interest: Yanzhuo Ye, Shasha Huang, Shahang Luo, Qianqian Liu, Hui Sun, Jiaqi Sun, Huisheng Liu declare that they have no conflict of interest.

Human and animal rights and informed consent: This article does not contain any studies with human or animal subjects performed by the any of the authors.

Compliance with Ethical Guidelines

The hiPSC cell line used in this study was purchased from UniXell Biotechnology (Cat.# iPSC-YS006-30, Bat.# 0520250606), and peripheral-blood-derived NK (PBNK) cells were obtained from Shenzhen Cell Valley Biopharmaceuticals (Cat.# SZCV2026-CV103, Bat.# GZSYS20260602). All work involving hiPSCs and PBNKs was reviewed and approved by the Institutional Review Board of Guangzhou National Laboratory.

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