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# Optimizing the procedure for manufacturing clinical-grade genetically manipulated natural killer cells for adoptive immunotherapy

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ABSTRACT

Background aims: Ex vivo–expanded natural killer (NK) cells hold significant potential as antitumor effector cells for adoptive immunotherapy. However, producing clinical-grade, genetically modified NK cells in sufficient quantities presents a considerable challenge.

Methods: We tested RPMI 1640, KBM581, SCGM, NK MACS, X-VIVO 15 and AIM-V, each supplemented with fetal bovine serum, human AB serum, human platelet lysate or Immune Cell Serum Replacement (SR) combined with feeder cells, to produce cytotoxic NK cells. Subsequent analyses were conducted to assess cell viability, expansion folds, cytotoxicity, immunophenotype and transcriptome profile of NK cells under certain conditions. Furthermore, transfer plasmids varying in transgene size, promoter elements, backbones and packaging plasmids with different envelopes were used to transduce NK cells, and differences in transduction efficiency were compared. Nucleofection was performed every 2 days from day 0 to day 12 to determine the optimal time window for gene editing.

Results: NK cells cultured in KBM581 medium supplemented with serum replacement exhibited the best expansion, achieving greater than 5000-fold increase within 2 weeks and exceeding 25 000-fold expansion within 3 weeks. In addition, NK cells cultured in KBM581 medium with human AB serum demonstrated the greatest cytolytic activities and exhibited greater expression of NKp30, 2B4, PRF1, granzyme B and IL2RG. Baboon envelope pseudotyped lentivirus outperformed baboon envelope-vesicular stomatitis virus type G hybrid envelope lentivirus, achieving robust NK-cell transduction. In addition, efficient gene knockout efficiency was achieved in NK cells on day 4 to day 6 post feeder cell activation using the LONZA DN-100 program, which can strike a balance between editing efficiency and cell expansion.

Conclusions: This research presents a Good Manufacturing Practice–compliant protocol using a feeder cell expansion system for the large-scale production of highly cytotoxic NK cells. The protocol facilitates genetic modification of these cells, positioning them as promising candidates for universal therapeutic applications in immunotherapy.



## Background

Chimeric antigen receptor (CAR) T-cell therapy involves modifying patient-derived T cells with CAR to target and eliminate malignant tumor cells. However, it encounters significant limitations during cancer treatment, especially allogeneic conditions [1,2]. Natural killer (NK) cells are innate immune lymphocytes capable of recognizing and targeting tumor cells without previous sensitization, which offer inherent advantages for the development of universal cell therapy products for cancer treatment [3]. Notably, NK cell infusion had been associated with complete remission in patients with acute myeloid leukemia (AML) with a poor prognosis, showing favorable tolerability with no graft versus-host-disease or autoimmune disease [4]. A more refined approach involving autologous or allogeneic NK cells for adoptive transfer has been applied in treating

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patients with ovarian, breast, renal cell cancer, glioblastoma and other solid tumors, yielding occasional clinical responses [5]. Furthermore, CAR-NK cell therapy has emerged as a promising approach in the treatment of hematologic malignancies [6]. However, technical and biological challenges associated with clinical-grade manufacturing of genetic modified NK cells have significantly tempered this approach.

With NK cells constituting only 5–15% of all peripheral blood lymphocytes, obtaining sufficient Good Manufacturing Practice–compliant NK cells for clinical application is challenging because of their low abundance and poor proliferation efficiencies in vitro. Various protocols have been developed to expand NK cells before clinical application. Initially, cytokines such as interleukin (IL)-12, IL-15 and IL-18 were used to expand NK cells, but these have been increasingly replaced by feeder cells, which were engineered from the K562 cell line to express membrane-bound forms of IL-15 or IL-21, along with CD137 ligand, CD64 and CD86 [5]. Furthermore, commercial media and supplements were developed for greater NK-cell expansion in recent years, but consensus is lacking on the most suitable option for clinical use. How to combine the culture medium, supplements and feeder cells to facilitate the expansion of NK cells deserves further exploration.

Genetic modifications aimed at enhancing the persistence, cytotoxicity, tumor-targeting and homing capabilities are the driving force in improving NK-cell immunotherapy [7]; however, inherent resistance to gene manipulation poses a significant hurdle. Viral transduction, successfully used for T cells, has been associated with low levels of transgene expression and unfavorable effects on cell viability when used with NK cells. During the viral transduction process, plasmid backbones, promoter elements, transfer gene size, viral envelopes and NK activation time may affect the transduction efficiency. Detailed electroporation protocols for efficient gene editing in NK cells need to be established because electroporation procedures, ribonucleoprotein (RNP) dosage, solution buffer composition and NK activation time may affect the gene-editing efficiency.

Here, we present a robust clinical-grade platform for manufacturing genetically manipulated NK cells for adoptive immunotherapy. Through comprehensive comparison of NK cell yield and functionality in various media and supplements, KBM581 medium supplemented with serum replacement (SR) and human AB serum enables optimal expansion and cytolytic activity of NK cells after 2 weeks of activation, respectively. Immunophenotypic analysis by multiparameter spectral flow cytometry and transcriptome sequencing shows that cytolytic NK cells express greater levels of CD57, NKp30, 2B4, PRF1 and IL2RG and greater activation of the AMP-activated protein kinase signal pathway. Genetic modifications, including CAR transduction and indicated gene knockout, were performed to identify the optimal constructs of transfer plasmids and gene-editing time windows for feeder cell activated NK cells. These efforts contribute to advancing the clinical translation of NK cell-based immunotherapy.

## Methods

### Cell culture

The K562 cell line (chronic myeloid leukemia cells) was obtained from ATCC (Manassas, VA, USA) and engineered to express luciferase by transfection with lentiviral vectors, resulting in the cell line named K562-luci. These cells were cultured in RPMI 1640 medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco/Thermo Fisher Scientific). Human embryonic kidney cells (HEK-293) were obtained from ATCC and cultured in Dulbecco's Modified Eagle's Medium (Gibco/Thermo Fisher Scientific) supplemented with 10% (v/v) FBS.

NK and T cells were isolated from human peripheral blood mononuclear cells obtained from Milestone Biotechnologies (Shanghai, China) using the NK Cell Isolation Kit (Miltenyi Biotec, Cologne, Germany) and Pan T cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. Both T cells and NK cells were cultured in G-Rex plates (Wilson Wolf, New Brighton, MN, USA). T cells were activated using CD3/CD28 Dynabeads (Gibco/Thermo Fisher Scientific) and cultured in CTS media (Gibco/Thermo Fisher Scientific) supplemented with 10% (v/v) FBS (Gibco/Thermo Fisher Scientific), 1% (v/v) L-Glutamine (Gibco/Thermo Fisher Scientific) and 200 IU/mL IL-2 (Beijing ShuangLu Pharmaceutical Co., Ltd., Shanghai, China), as previously described [8]. Freshly isolated NK cells were activated using m21-K562 feeder cells (Hangzhou Zhongying Biomedical Technologies, Hangzhou, China). The m21-K562 cell line serves as an NK-cell expansion and activation agent, engineered to overexpress membrane-bound IL-21, CD14, CD19, CD86 and CD137 on its surface. When used in conjunction with IL-2 in vitro, this system selectively expands and activates NK cells at a ratio of 1:2. The expansion and activation were conducted in various culture media, including RPMI-1640, KBM581 (Corning, New York, NY, USA), SCGM (CellGenix, Freiburg, Germany), NK MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), X-VIVO 15 (Lonza, Basel, Switzerland) or AIM-V (Gibco/Thermo Fisher Scientific). All media were supplemented with 5% (v/v) FBS, human AB serum (Sigma-Aldrich, St. Louis, MO, USA), human platelet lysate (HPL; BioInd, Beit Haemek, Israel), or Immune Cell Serum Replacement (SR) (Gibco/Thermo Fisher Scientific). IL-2 was added to achieve a final concentration of 200 IU/mL. On day 0, $3 \times 10^5$ NK cells and feeder cells were suspended in 8 mL of the corresponding complete growth medium. The plate remained stationary until day 4, when one half of the medium was replaced with fresh medium. The culture continued until day 7, at which point $1 \times 10^6$ NK cells were harvested and transferred to another 8 mL of fresh medium for further culture. The same procedure as day 4 was repeated on day 11. On day 14, cells were harvested for functional testing, and the remaining cells were maintained at $1 \times 10^6$ in 8 mL of fresh medium for continued culture until day 21.

### B2M gene knockout in NK cells

NK cells were harvested, washed once with phosphate-buffered saline and prepared for electroporation. RNP complexes were assembled immediately before electroporation by combining 7 μg of Cas9 protein (Kactus Biosystems, Shanghai, China) with 3 μg of sgRNA targeting B2M (sequence: CGTGAGTAAACCTGAATCTT, synthesized by Genscript, Nanjing, China) and incubating the mixture at room temperature for 15 minutes. The cell-RNP mixture was then transferred to an electroporation cuvette and subjected to electroporation using the DN-100 program on a 4D-Nucleofector (Lonza). After electroporation, NK cells were incubated in pre-warmed medium and cultured further.

### CAR construction and virus production

The CAR construct comprised a target-specific single-chain variable fragment (scFv), a CD8α hinge region, either a 4-1BB or CD28 co-stimulatory domain, and a CD3ζ signaling domain. A switch of herpes simplex virus thymidine kinase and secreted IL-15 were linked with the CAR fragment through a T2A sequence in some vectors. These sequences were cloned into different plasmid vectors with various promoters and gene elements.

Lentivirus was produced in HEK-293T cells using baboon envelope (BaEV) or a hybrid BaEV-vesicular stomatitis virus type G (VSVG) envelope expressed packaging and CAR gene transfer plasmids, following established protocols [9]. To summarize, $1.5 \times 10^7$ HEK-293T cells were seeded in 15-cm dishes and cultured for 24 hours. For transfection, the medium was replaced with a mixture of 40 μg of plasmid DNA and 80 μL of Lipofectamine 3000 per dish, followed by the addition of fresh medium. After 60–72 hours, the cell supernatant was collected, filtered through a 0.45-μm filter and concentrated

by centrifugation at 30 000g at 4°C, for 3 hours. Lentivirus titration was performed on HEK-293T cells using serial dilutions. NK cells were infected with lentivirus at a multiplicity of infection of 5 on day 4, whereas T cells were infected on day 2. After 48 hours postinfection, lentivirus was removed, and fresh culture medium was added. Transfection efficiency was assessed by flow cytometry 5 days post-transfection.

### CD107a degranulation assay

NK and K562 cells were co-cultured at a 1:1 ratio in U-bottom 96-well plates. Monensin Solution (BD Biosciences, Franklin Lakes, NJ, USA) and PE/Cyanine7-conjugated CD107a antibody (clone H4A3; BioLegend, San Diego, CA, USA) were added to each well following the recommended dosages. The cells were then incubated at 37°C with 5% $CO_2$ for 4 hours. After incubation, cells were harvested from the wells for antibody staining, and flow cytometry was used to assess the surface expression of CD107a in $CD56^+$ NK cells.

### In vitro cytotoxicity assays

NK and K562-luci cells were seeded in triplicate in a white opaque plate at indicated ratios (ranging from 1:1 to 1:8) in 200 mL of medium. The plates were then incubated at 37°C with 5% $CO_2$ for 4 hours and 24 hours. K562-luci cells alone were incubated as a control. Luminescence detection was performed using the luciferase assay system (Promega, Madison, WI, USA) following the manufacturer's instruction.

### Flow cytometry

FITC-conjugated human CD5 protein (Acro, Newark, DE, USA) was used to detect the expression of anti-CD5 CAR. FITC-conjugated CD3 antibody (clone SK7; BioLegend), BV421-conjugated CD16 antibody (clone 3G8; BioLegend) and APC-conjugated CD56 antibody (clone HCD56; BioLegend) were used to gate NK cells. To identify the immunotype of NK cells in different culture media, two antibody panels were designed and provided in Supplementary Table 1. NK cells were stained with different antibodies and analyzed using a multiparameter spectral flow cytometer (CYTEK, NC-CLC). Intracellular staining of NK cells was performed according to the BD Fixation/Permeabilization Kit (BD Biosciences) instructions.

### Cytokine analysis

Cultured supernatants of NK cells and K562 cells co-incubated for 24 hours were collected and stored at −80°C. Cytokine detection was carried out using Luminex (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Data analysis was performed using MILLIPLEX Analyst software (Version 5.1; Merck Millipore, Burlington, MA, USA).

### RNA sequencing

NK cells were collected and preserved in Trizol at −80°C. Total RNA extraction was conducted using the RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Sequencing and analysis were performed by Wuhan Aiji Baike Biotechnology Co., Ltd. Differential gene expression was determined using a threshold of false diacovery rate (FDR) < 0.05 and |logFC| > 1.

### Graphs and statistical analysis

Graphs and data analyses were performed using GraphPad Prism (8.3.0; GraphPad, San Diego, CA, USA) and SPSS (26.0; IBM Corp, Armonk, NY, USA). Unless specified otherwise, all data represent results from at least three independent experiments and are presented as mean § standard deviation. Statistical significance was determined using one-way analysis of variance, two-way analysis of variance, Student's t-test, or log-rank test. P values are indicated as follows: not significant (ns), *P < 0.05, **P < 0.01, ***P < 0.001, or ****P < 0.0001.

## Results

### Expansion of clinical-grade NK cells in different media

To achieve large-scale expansion of clinical-grade NK cells ex vivo, we developed a total of 24 kinds of culture formulations by combining six kinds of commercial media with four kinds of supplements (Figure 1A). NK cells were cultured in 24-well Grex according to the schematic diagram shown in Figure 1B; no other cytokines were applied during this process with an exception of IL-2. Notable proliferation difference was observed between various culture formulations. At day 14, KBM581 supplemented with SR (2D, KBM581-SR) exhibited the greatest expansion (5217.65 § 370.90), followed by KBM581-HPL (2C, 2866.23 § 26.51), KBM581-FBS (2A, 2850.17 § 26.51), NK MACS-human AB serum (4B, 2395.70 § 17.96) and KBM581-human AB serum (2B, 2383.90 § 17.41), all of which expanded more than 2000-fold. Four groups with the lowest expansion were AIM-V-SR (6D, 7.40 § 1.66), RPMI-SR (1D, 12.80 § 1.48), NK MACS-SR (4D, 57.63 § 0.61) and SCGM-SR (3D, 104.93§2.34) (Figure 1C). These results highlight that KBM581 and NK MACS media have stronger potential to support NK-cell expansion. Specifically, KBM581 medium combined with SR achieved the greatest expansion, although SR combined with other media showed lower expansion abilities.

At day 21, the top five groups with the greatest expansion were NK MACS-human AB serum (4B, 56279.81 § 911.99), NK MACS-HPL (4C, 55341.71 § 227.81), KBM581-SR (2D, 25411.19 § 1628.97), KBM581-FBS (2A, 17999.55 § 424.24) and SCGM-HPL (3C, 15876.64 § 903.31), whereas the five worst groups for proliferation were AIM-V-SR (6D, 8.73 § 2.90), RPMI-SR (1D, 9.71 § 1.14), NK MACS-SR (4D, 181.64 § 2.23), NK MACS-FBS (4A, 216.13 § 4.87) and SCGM-SR (3D, 475.71 § 32.10) (Supplementary Figure 1). Groups that exhibited poor expansion at day 14 continued to show limited growth at day 21. Notably, the NK MACS-FBS group, which demonstrated robust expansion at day 14 (1418.98 § 276.68), experienced a decline in cell numbers during the following week, resulting in overall poor expansion (216.13 § 4.87). This trend suggests the onset of cellular senescence or exhaustion within this group.

The viability of NK cells was stable and consistently greater than 90% in the top eight best-expanded groups, except in the KBM581-human AB (2B) and NK MACS-FBS (4A) culture systems where it was lower than 90% at day 21 (Figure 1D). In addition, the proportion of T cells ($CD3^+CD56^-$) did not exceed 2%, and the proportion of NK cells ($CD3^-CD56^+$) was greater than 95% in all groups (Figure 1E). This suggests that NK cells can be massively expanded in an animal serum-free system.

### Functional characterization of NK cells in different culture systems

At day 14, NK cells of eight groups (2A, 2B, 2C, 2D, 3B, 4A, 4B, 4C) with the greatest expansion were harvested to assess their functional characterization. When different groups of NK cells were co-incubated with K562 cell line, NK cells cultured with KBM581, especially 2B group, showed enhanced CD107a degranulation release (Figure 2A) and tumor-killing capacity, regardless of brief (4 hours) or prolonged (24 hours) co-incubation (Figure 2B).

To analyze the cytokine secretion patterns of NK cells in eight groups, supernatants from co-culture medium were collected and performed using Luminex. Overall, NK cells cultured with KBM581

and SCGM media secreted more cytokines and chemokines compared with those cultured with NK MACS. Among all tested groups, NK cells from the 2D group exhibited the greatest cytokine secretion capacity, whereas the 2B serum group displayed the strongest cytotoxicity-related cytokines (Figure 2C). When supplemented with FBS or human AB Serum, NK cells harvested from KBM581 medium (2A and 2B) exhibited stronger cytotoxicity-related factors compared with those from NK MACS medium (4A and 4B). Comparison of cytokine release during NK cell encounters with tumor cells revealed that greater levels of CCL3 CCL4, CXCL10 and IL-8 were secreted by NK cells with greater cytotoxicity. Significant differences in interferon (IFN)-g and tumor necrosis factor (TNF)-a secretion were also observed only when using human AB Serum as supplement (Figure 2D). In other groups of NK cells with relatively high cytotoxicity, the secretion levels of these two cytokines were greater correspondingly (Supplementary Figure 2).

## Immunophenotype of NK cells among different groups

To further characterize the immunophenotype of efficiently expanded and tumor-killing NK cells, multiparameter spectral flow cytometry–based assays were performed. Two antibody panels covering NK cell differentiation lineage, receptor repertoire and functional capacity were designed, followed by tSNE clustering and visualization. NK cells from KBM581-SR (2D) exhibited significantly different clustering characteristics compared with the other groups. In panel one, which included 20 biomarkers, NK cells were clustered into eight populations (Pop0-Pop7) to analyze their relative distributions across groups (Figure 3A). The abundances of Pop2 and Pop3 were significantly greater than in other groups, characterized by a notable decrease in CD16, CD7 and CD57 positive populations, reduced NKG2C and NKG2A expression and mild enhanced NKp44 expression (Figure 3, B–C and Supplementary Figure 3A). In panel two, which includes 15 antigen markers, NK cells were also divided into eight subgroups (Pop0-Pop7). The abundances of Pop0 and Pop7 in NK cells cultured in 2D were greater (Figure 3D), both characterized by CD16 negativity (Figure 3, E–F and Supplementary Figure 3B). In addition, NK cells cultured in 2B expressed greater levels of Ki67, CD127, and granzyme B (Figure 3E), whereas the $CD16^{-}CD39^{+}$ and $CD16^{-}CD25^{+}$ populations were more prevalent in 2D-cultured NK cells compared with other groups (Figure 3F).

## Transcriptome analysis of NK cells among different groups

To further explore the intrinsic characteristics of NK cells cultured under different groups, RNA transcriptome sequencing analysis were performed. Integrated analysis of gene expression across all groups revealed that NK cells cultured in KBM581 exhibited greater expression of both activating (NKp30, NKp44, NKp46, 2B4, KIR2DS4, NKG2C, CD27, etc.) and inhibitory (CBLB, CBL, CISH, LAG3, TIGIT and CD96) receptors or related signals compared with NK cells in NK MACS media. Specifically, NK cells cultured with 2B showed the strongest expression of PRF1, certain activating NK cell receptors (NKp30, 2B4), cytokine receptor (IL2RG) and AMPK signal. NK cells from 2D exhibited greater expression of some cytokines (IL-15, IFN-g and TNF-a), specific activating NK-cell receptors (NKp44), transcriptional activator (IRF1) and key enzymes (IDH1) or transporters (GLUT1) involved in energy metabolism. However, more critical metabolic enzymes and transporters (ACSY, FASN, PDHA1, SLC16A1) were expressed at greater levels in NK cells derived from NK MACS media (Figure 4A). The differential expression of these genes partially explains the variations in NK-cell expansion and functionality under different culture groups.

By comparing the transcriptome levels of NK cells cultured in KBM581 and NK MACS, 62 upregulated genes and 107 downregulated genes were identified under indicated supplements (Figure 4B). These co-upregulated genes were enriched in pathways related to cell proliferation and apoptosis (including PIMREG, AREG, MYC, KLF9, PPARGC1B, ZNF485, TFAP4, FOSL1) as well as ribosome biogenesis and assembly (NOP16, BOP1, RRS1, RRP12, EXOSC5). Among the co-downregulated 107 genes were immune checkpoint receptors (TIGIT, CTLA4, LAG3, SLAMF1, CD200R1) and numerous cell adhesion-related molecules (ITGA1, CD82, PPFIBP1, VSIG10, THBS1, TMEM98, CADM1, ITGB5) (Supplementary Table 2). Regardless of whether KBM581 or NK MACS media was used, NK cells showed 18 co-upregulated genes when supplemented with human AB serum and 11 co-upregulated genes when supplemented with HPL, compared with NK supplemented with FBS. The shared genes among these include ITGA4, EPAS1, AREG, PRKCE, HIC1, BOLA2B, HOXB9 and LGMN, primarily involved in regulating cell proliferation, apoptosis, and response to hypoxia (Figure 4C). Regarding downregulated genes, NK cells cultured with human AB serum showed a shared downregulation of 18 genes, whereas those supplemented with HPL exhibited a shared downregulation of nine genes. The overlapping genes include ABCG1, ITGB5, GREM2, PI16 and P2RY8-2, which are associated with lipid metabolism and intercellular signaling pathways (Figure 4D).

## Optimization of transfection and gene-editing for NK cells

Traditional VSVG enveloped lentivirus, typically effective for T cells, is ineffective in NK cells because of the absence of corresponding receptors. To overcome this obstacle, we sought to enhance transfection efficiency in NK cells by optimizing plasmid backbones, promoters, and viral envelopes (Figure 5A). Initially BaEV-VSVG hybrid enveloped lentivirus with the same plasmid backbone and CAR structure were employed; four transfer plasmids named 1261, 1262, 1263 and 1264 achieved greater than 50% transfection efficiency in T cells, with the EF-1a promoter demonstrating the greatest expression, significantly surpassing the other three kinds of lentiviral. However, the transfection efficiency was less than 10% in NK cells (Figure 5B). Subsequently, we modified the plasmid backbone by replacing the original pCDH vector with pLKO (1265) and pLVX (1266). In these updated virals, we substituted the traditional single-chain variable fragment with VHH, adjusted the co-stimulator and introduced herpes simplex virus thymidine kinase as a molecular switch. Despite these modifications, the transfection efficiency of the new viruses in both T and NK cells remained less than 10% (Figure 5C), potentially because of the large size of transfer plasmids. Then, BaEV enveloped viral were applied and robust transduction were observed in both T and NK cells, with even greater transfection efficiency in NK cells (Figure 5D).

During the cell culture process, we sought to determine the time window for efficient gene editing of NK cells while maintaining efficient expansion. By using P3 buffer and DN-100 program, NK cells were nucleofected every 2 days from day 0 to day 12, preceding NK cell activation on day 0. To avoid potential interference with cell expansion caused by gene deficiency, B2M gene were selected as a target. The knockout efficiency exceeded 60% at each time point under indicated time point (Figure 5E). Interestingly, the efficiency in pre-activated NK cells was lower compared with post-activated groups ($P < 0.0001$). As the activation period of NK cells was extended, the knockout efficiency demonstrated an increasing trend, peaking on day 10 at nearly 90%, then declined gradually (Figure 5E).

Nucleofection had a notable impact on the proliferation and viability of NK cells. NK cell viability was at its lowest, and cell numbers were minimal when harvested on day 2 after electroporation, leading to ineffective expansion throughout the culture period. The viability of NK cells electroporated at other time points was able to recover to greater than 90% on day 14 (Figure 5F). With the exception of the day 2 knockout group, cells electroporated during the first week exhibited expansion multiples exceeding 150-fold within 14 days, with the day 6 knockout group achieving the highest expansion (369.35 §

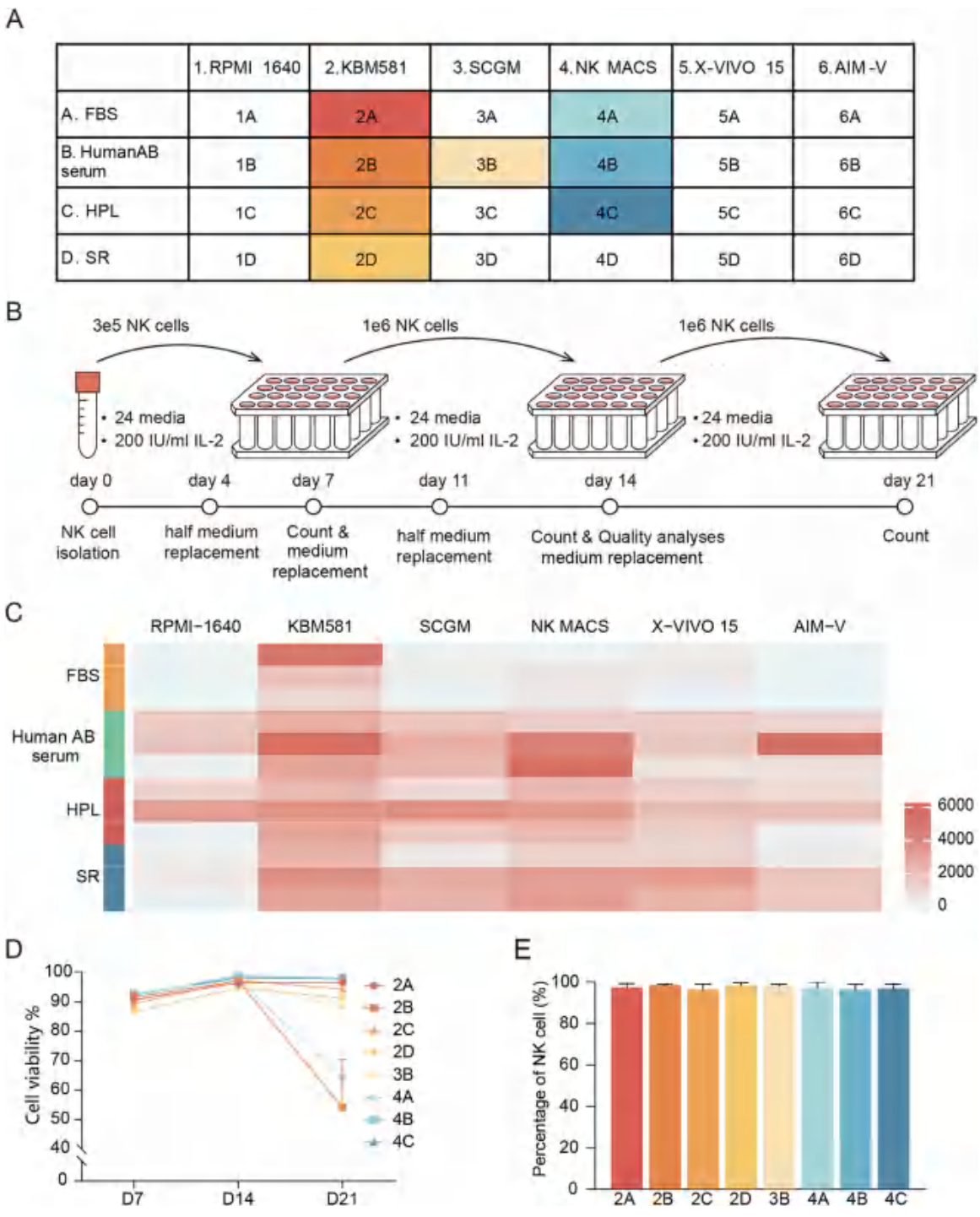


Fig. 1. Expansion of NK cells derived from frozen PBMC in different media. (A) Combination of commercial culture media and supplements used in this research. (B) Schematic diagram of NK cell expansion ex vivo. (C) NK cell expansion at day 14 in all test groups. The expansion in group 2C was significantly superior to that in 1A ($P = 0.0330$), 1D ($P = 0.0168$), 3D ($P = 0.0249$), 5D ($P = 0.0401$) and 6D ($P = 0.0147$). In addition, the amplification in group 4C was significantly better than that in groups 1D ($P = 0.0438$) and 6D ($P = 0.0387$). Groups 2A, 2B, 2D, 3B, 4A and 4B showed greater amplification compared with the remaining groups, but the differences were not statistically significant. This represents the expansion profile of NK cells from one representative donor. In this study, NK cells from three donors were investigated. (D) Viability of NK cells in the best-expanded eight groups. (E) The percentage of $CD3^{-}CD56^{+}$ NK cells in top eight groups for optimal expansion.

34.50). However, the proliferation of all groups in the second week did not exceed 80-fold (Figure 5G). Taking both knockout efficiency and expansion folds into consideration, the optimal gene editing time window was identified to be from day 4 to day 6 after NK-cell activation.

## Discussion

NK cells play a critical role in the human immune system by bridging innate and adaptive immunity. Their excellent safety profile and potent cytotoxicity have attracted significant attention in cellular

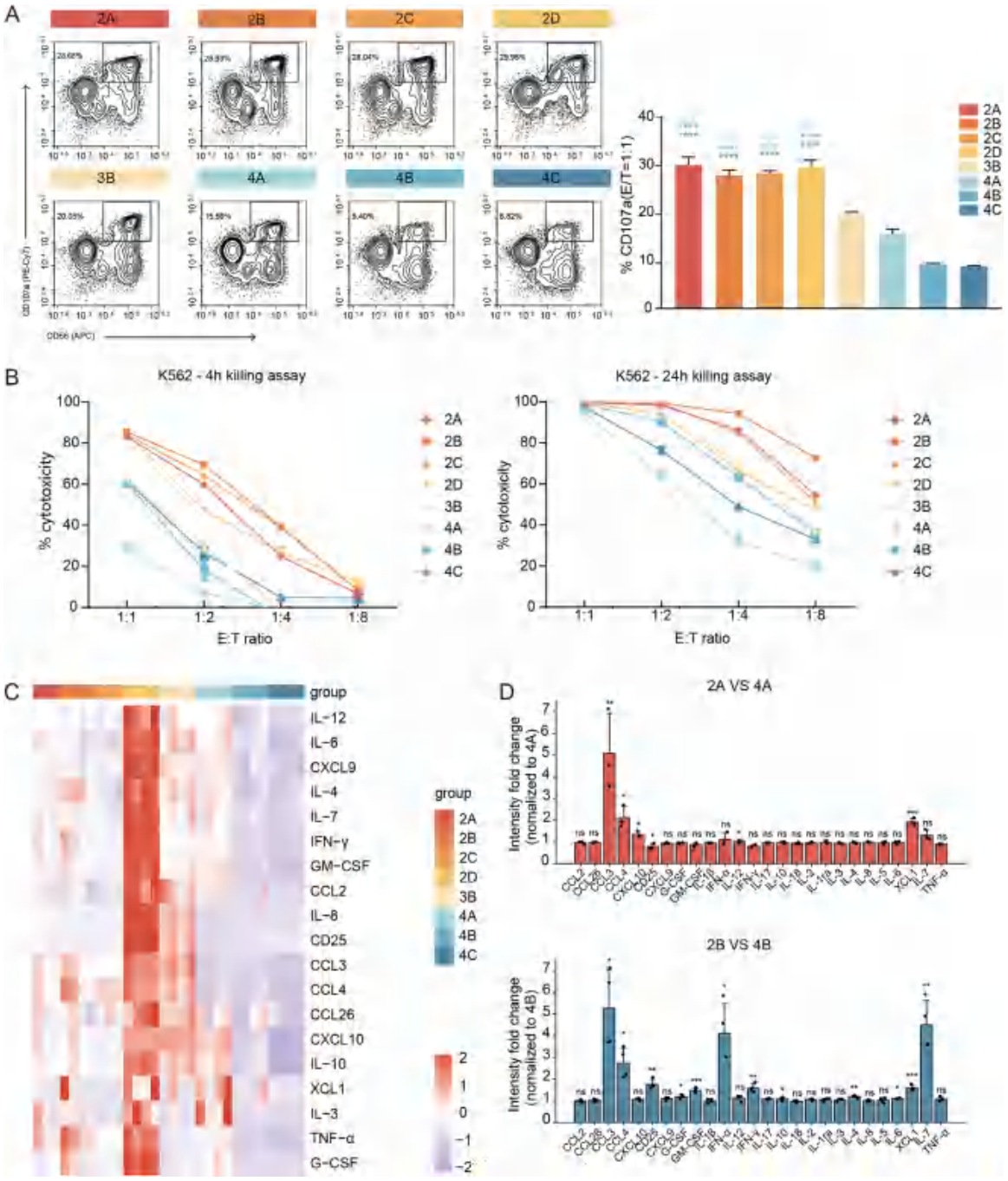


Fig. 2. Functional characterization of NK cells in top eight groups for optimal expansion. (A) Representative CD107a degranulation of NK cells from indicated culture groups, flow cytometry plot (left) and corresponding statistics (right). (B) NK-cell cytotoxicity against K562-luciferase cells at indicated effector-to-target ratios, as determined by viable cell numbers following 4-hour (left panel) and 24-hour (right panel) co-culture periods. Error bars show mean § standard error of the mean. (C) Heatmap displaying cytokine secretion levels by NK cells. (D) Analysis of differential cytokine secretion between two groups with different cytotoxicity (KBM581-FBS versus NK MACS-FBS and KBM581-human AB serum versus NK MACS- human AB serum).

immunotherapy [10]. Infusion of donor-derived NK cells in patients has shown promise in inducing remission and preventing relapse in refractory AML [11]. Similarly, infusing donor-derived NK cells in patients with relapsed myeloid malignancies post-transplantation can re-induce remission [12]. Notably, none of these clinical trials reported severe graft versus-host-disease or other adverse effects. In addition, CAR-NK cells have demonstrated enhanced antitumor activity. For instance, umbilical cord blood–derived NK cells engineered with anti-CD19 CAR exhibit potent yet mild antitumor effects against B-cell malignancies. Recent studies have shown that human leukocyte antigens genotype matching is no longer a limiting factor for the source of NK cell donors [13]. However, infusion doses of cells in these trials were all greater than 1 £ $10^7$/kg, significantly greater than typical doses for CAR-T cell therapy [11–13]. Considering the potential for repeated infusions to enhance efficacy or reduce costs through universal cell therapy, there is an increased demand for large-scale, clinical-grade NK cell manufacturing.

NK-cell expansion protocols described in previous reports have not fully met the objective requirements. Four kinds of commercial media including RPMI, SCGM, NK MACS and Tex MACS were compared, and NK MACS achieved the greatest expansion with approximately 900-fold within 21 days when supplemented with 5% human

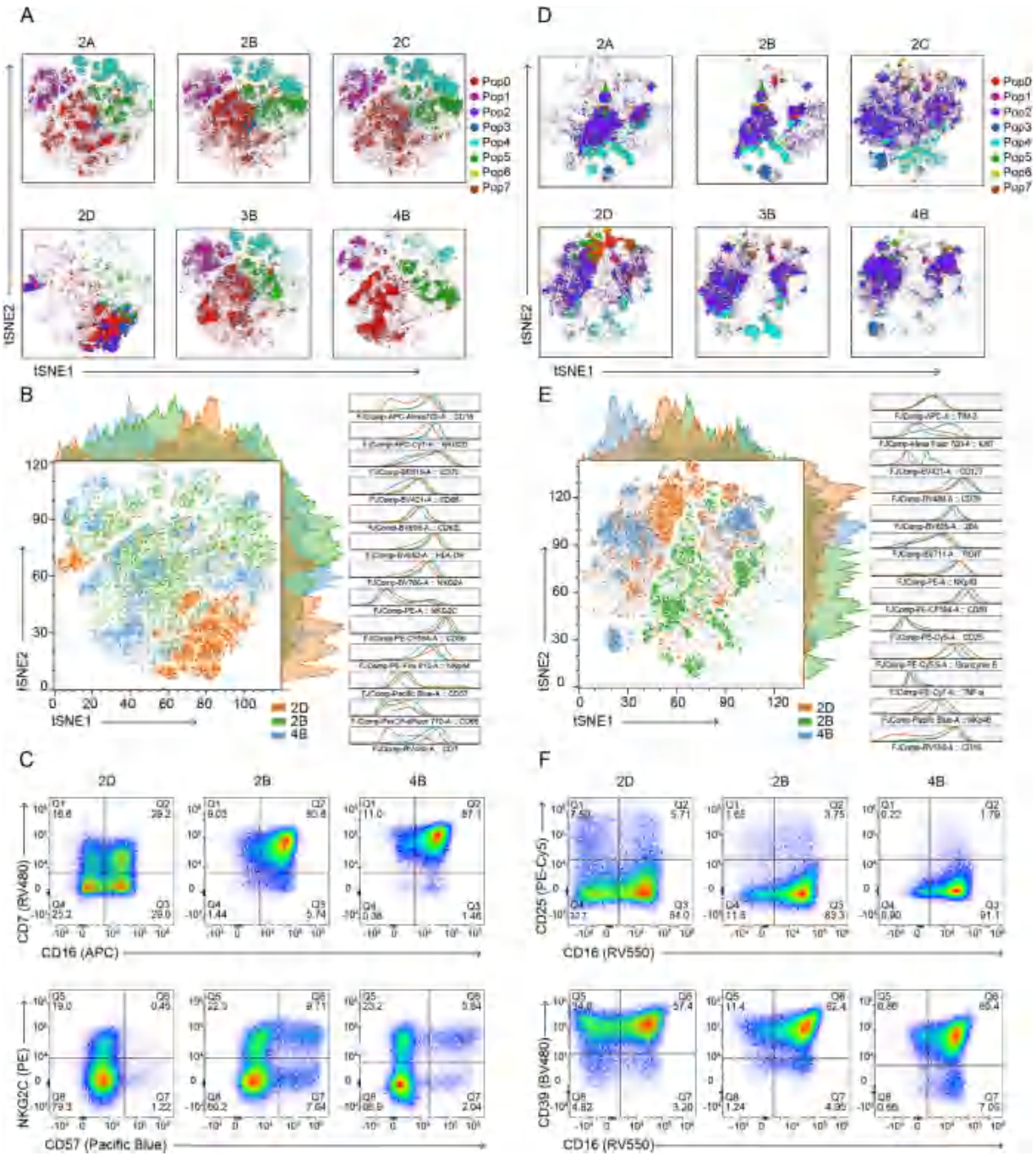


Fig. 3. Immunophenotype of NK cells in different groups. (A) tSNE plot of NK cells from six groups in panel 1; (B) Overlaid tSNE plot and peak diagram of specific markers of NK cells from 2D (orange), 2B (green) and 4B (blue) groups in panel 1. (C). Scatter plot showing the expression of CD16, CD7, CD57 and NKG2C in NK cells from the 2D, 2B and 4B groups in panel 1. (D) tSNE plot of NK cells from six groups in panel 2. (E) Overlaid tSNE plot and peak diagram of specific markers of NK cells from 2D (orange), 2B (green) and 4B (blue) groups in panel 2. (F) Scatter plot showing the expression pattern of CD16, CD25 and CD39 in NK cells from the 2D, 2B and 4B groups in panel 2.

AB serum [14]. Without serum and feeder cells, NK MACS medium demonstrated reasonable performance, but its expansion rate did not exceed 200-fold within 20 days, falling short of clinical requirements [15]. When using cord blood as the cell source, expansion fold within 14 days using SCGM medium and human AB serum was 3127 [6]. Despite advancements in NK-cell–production techniques, comprehensive comparisons among commonly used culture media are still in demand, and there is a need to identify serum substitutes to address safety and supply limitations. Our protocol indicates that KBM581 and NK MACS are the most suitable culture media for NK cell growth (Figure 1). KBM581 paired with SR achieved an average expansion of NK cells by 5218-fold within 14 days (Figure 1C), representing the greatest expansion rate within this time frame. Moseman et al. [16] demonstrated that AIMV+SR medium yielded superior expansion results, significantly surpassing those observed in the present study. This discrepancy could be attributed to variations in culture systems and donor-specific factors. By the end of the third week, KBM581-SR reached an expansion fold of 25411(Supplementary Figure 1), ranking third overall. NK MACS performed best when combined with human AB serum, that is consistent with previous reports. At 14 days, the expansion fold was 2396-fold, but by day 21, it significantly increased to 56280-fold, ranking first among all

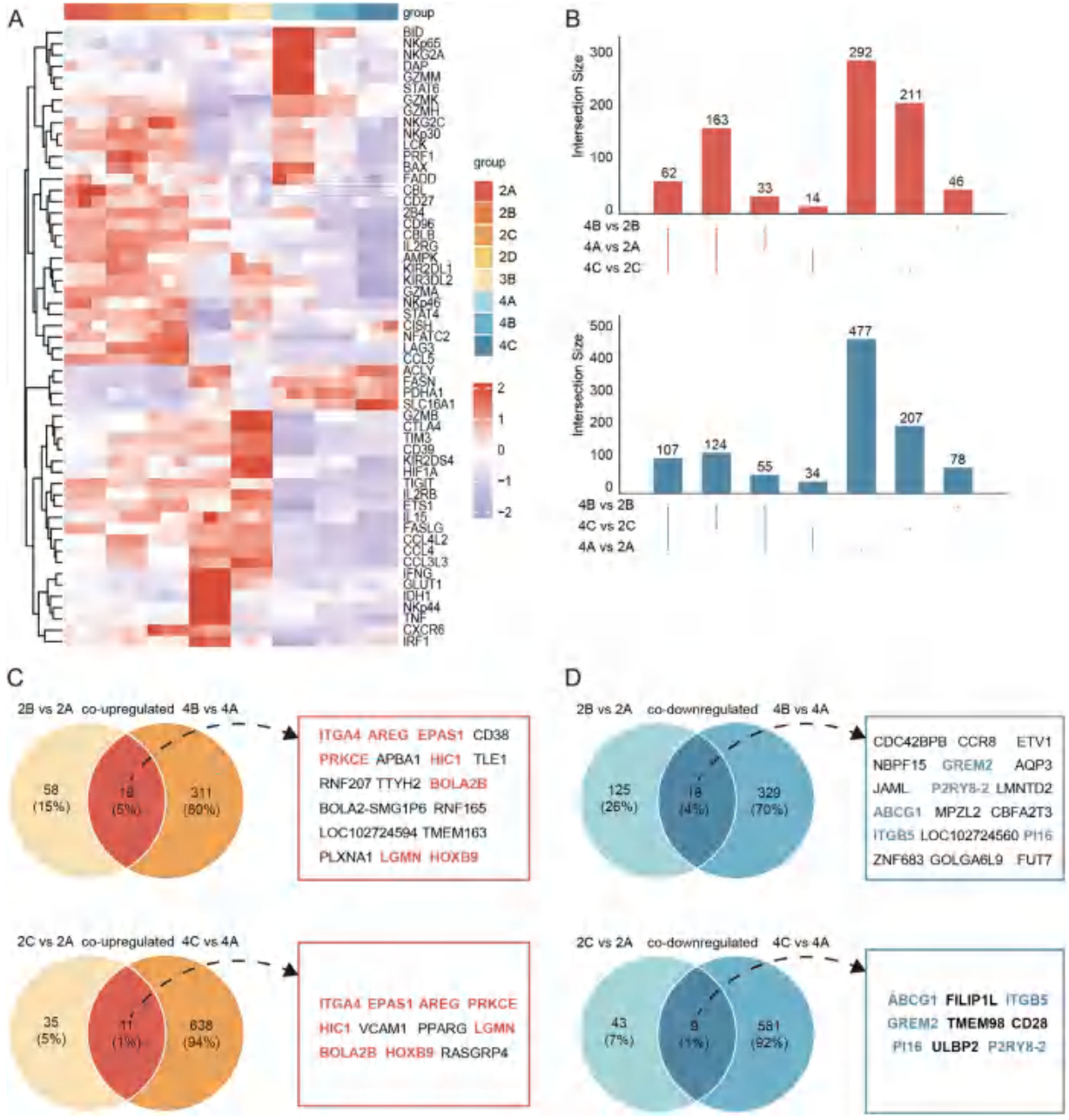


Fig. 4. Transcriptome analysis of NK cells in in top eight groups for optimal expansion. (A) Heatmap of differential gene expression in NK cells from eight groups. (B) Waterfall plots showing differentially expressed genes in NK cells cultured with NK MACS and KBM581 media (top: upregulated; bottom: downregulated). (C) Genes upregulated in NK cells cultured with human AB serum and HPL were compared FBS, with shared genes highlighted in red. (D) Genes downregulated in NK cells cultured with human AB serum and HPL were compared with FBS, with shared genes highlighted in blue.

groups. Similar performance was observed with NK MACS-HPL group, reaching 55341-fold by day 21. Notable differences were observed between NK cells harvested from these two media. Correspondingly, CD107a degranulation was also more pronounced in NK cells cultured in KBM581 medium (Figure 2A). Although both media yielded NK cells capable of achieving nearly 100% cytolysis at an effector-to-target ratio of 1:1 after 24 hours (Figure 2B), NK cells cultured in KBM581 demonstrated stronger cytotoxicity compared with NK MACS under lower effector-to-target ratios or shorter incubation times. NK cells cultured in KBM581-SR medium secreted greater levels of cytokines, despite lower cytotoxicity potency. The secretion of cytokines (IFN-γ, TNF-α, IL-8, etc.) and chemokines (CCL3, CCL4, CXCL10, etc.) suggests their potential for better expansion and homing in vivo, although further validation is needed.

The function of NK cells is controlled by a balance of various surface receptors, including inhibitory receptors such as NKG2A, CD96, TIGIT, KIR2DL1–KIR2DL3, KIR2DL5 and KIR3DL1–KIR3DL3 and activating receptors such as NKG2C, NKG2D, NKp30, NKp40, NKp44 and NKp46, as well as KIR2DS1–KIR2DS5 and KIR3DS1 [17]. Studies have shown that NK cells in patients with AML often exhibit enhanced NKG2A expression and decreased NKp46 expression, with this phenotype varying with disease status. Phenotypic analysis of NK cells and their ability to produce cytokines (such as TNF-α) can predict treatment response [18]. Multicolor flow cytometry was conducted to analyze the immunophenotype of NK cells in each group (Figure 3). Certain markers such as CD16, CD57, CD7, NKG2D and NKp44 showed altered expression levels in NK cells from indicated groups. Notably, upregulated

A

| number | envelope | viral vector | promoter | Binder | Co-stimulator | Switch | IL-15 |
|---|---|---|---|---|---|---|---|
| 1261 | VSVG & BaEV | pCDH | EF-1a (212bp)-LTR (269bp) | ScFV | CD28 | - | + |
| 1262 | VSVG & BaEV | pCDH | PGK(500bp) | ScFV | CD28 | - | + |
| 1263 | VSVG & BaEV | pCDH | SFFV(471bp) | ScFV | CD28 | - | + |
| 1264 | VSVG & BaEV | pCDH | EF-1a (1179bp) | ScFV | CD28 | - | + |
| 1265 | VSVG & BaEV | pLKO | EF-1a (1179bp) | VHH | 4-1BB | HSV-TK | + |
| 1266 | VSVG & BaEV | pLVX | EF-1a (1179bp) | VHH | 4-1BB | HSV-TK | + |
| 1267 | BaEV | pLVX | EF-1a (1179bp) | VHH | 4-1BB | HSV-TK | + |

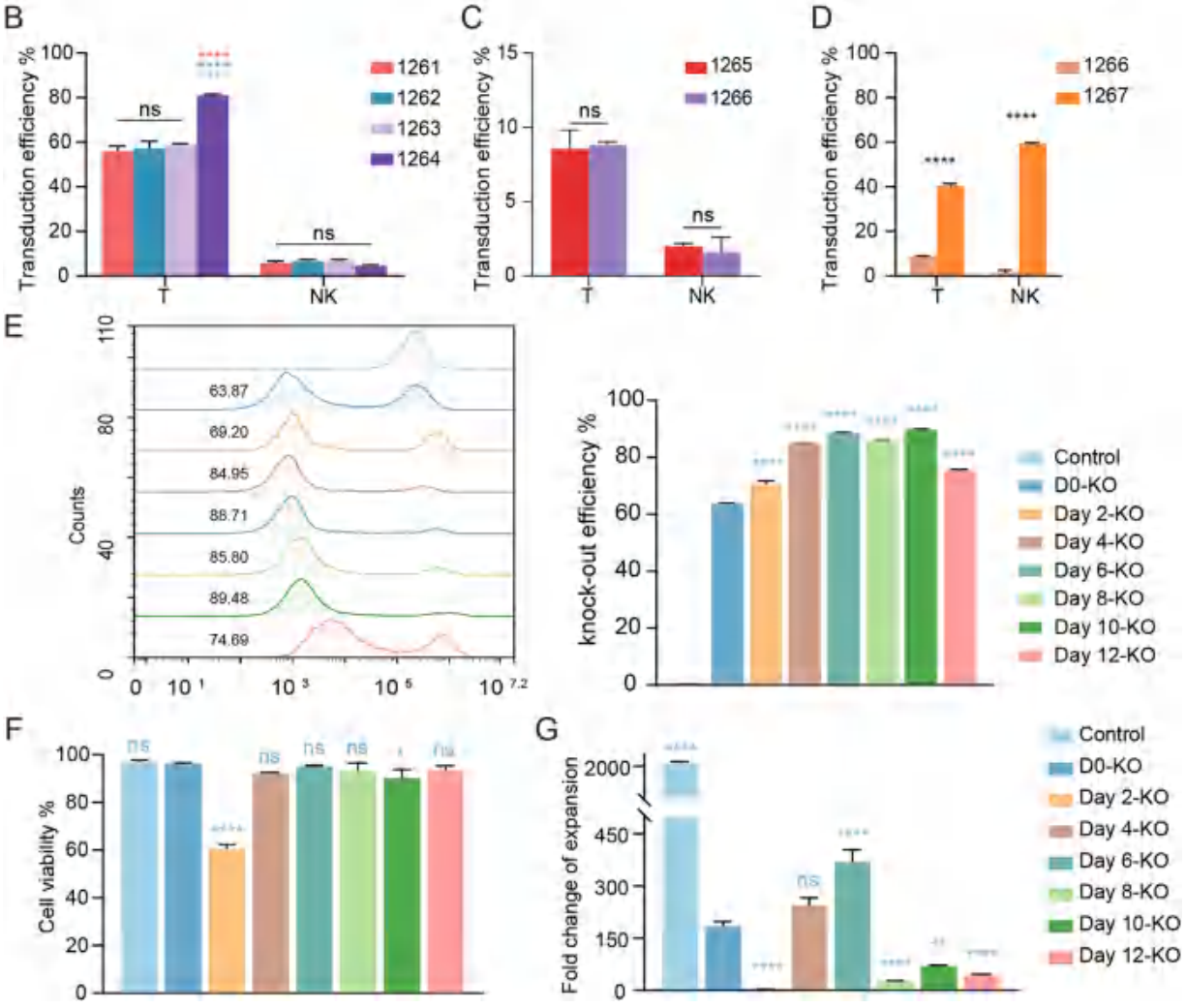


Fig. 5. Optimization of strategies for virus transduction and gene editing of NK cells. (A) Detailed information of transfer plasmid used for virus packaging. (B) Transduction efficiency of the CAR gene with different promoters in T and NK cells. (C) Transduction efficiency of transfer plasmid with different backbones in T and NK cells. (D) Transduction efficiency of viruses with different envelopes in T and NK cells. (E) Efficiency of gene editing at indicated time points in NK cells (left: representative flow cytometry plots; right: statistical graph). (F) Viability of NK cells after electroporation at indicated time points. (G) Expansion of NK cells after B2M gene knockout at indicated time points.

expression of NKp44 in NK cells from the 2D group was also evident in transcriptome analysis (Figure 3B).

CAR-NK cells that exhibit favorable responses in patients show enrichment of effector genes such as PRF1 and NK functional genes, along with genes related to chemokine signaling (CXCR6 and CMKLR1). These cells also demonstrate greater levels of oxidative phosphorylation and mitochondrial adaptability [13]. Conversely, CAR-NK cells from patients with less-favorable responses exhibit upregulation of genes associated with hypoxia (HIF1A, MAFF, JMJD6, DDIT3 and SIAH2), as well as immune inhibitory genes (IL10 and LAG3) [13]. RNA sequencing analysis of NK cells in different groups revealed that NK cells cultured in KBM581 exhibited stronger cytolysis in vitro and expressed greater levels of PRF1 and activation receptors (NKp30, NKp46, NKG2C) (Figure 4), indicating that these cells were in a highly activated effector state [19–21]. However, NK cells cultured in NK MACS demonstrated more sustained expansion, characterized by lower expression of hypoxia-related genes (HIF1A) and strong metabolic adaptability, particularly in fatty acid metabolism, lactate-to-pyruvate conversion and the tricarboxylic acid cycle (Figure 4). This indicates a potential metabolic advantage for NK cells

cultured in NK MACS, which may contribute to their sustained expansion and functional properties [22].

In gene manipulation of NK cells, viral infection and CRISPR/Cas9-mediated gene editing are the most commonly used methods [23]. Enhancing the efficiency of transduction and gene editing in NK cells is crucial for advancing NK-cell therapies. VSVG-enveloped lentivirus, widely used for traditional CAR-T cell preparation, exhibits very low transduction efficiency in NK cells (Figure 5), possibly because of the absence of corresponding receptors [7]. Studies suggest that transient blockade of TBK1/IKKε kinases can significantly improve the transduction efficiency of VSVG-enveloped virus in NK cells [24]. However, more evidence indicates that BaEV-enveloped virus holds greater potential in NK cells (Figure 5). To address challenges in viral yield and overcome limitations associated with lentiviruses, transition to retroviral might be a promising approach [25].

Editing NK cells with CRISPR/Cas9 presents a challenging technical hurdle. Referring to previous studies that have systematically compared different electroporation protocols and buffers, we used P3 buffers and the DN-100 program [26]. However, compared with nucleofection at 48 hours post-activation, we recommend performing this procedure at 4–6 days post-activation to achieve greater editing efficiency with minimal adverse effects on cell proliferation. This preference may stem from the use of two different NK-cell activation methods. The other approach used high concentrations of IL-2 and beads conjugated with anti-human NKp46 and anti-CD2 antibodies, whereas we used feeder cells derived from K562 cells. By day 2, the viability of NK cells performs the worst, with nucleofection during this period potentially detrimental to cell recovery.

## Conclusions

In summary, we have proposed a comprehensive optimized protocol that includes clinical-grade, large-scale expansion of gene-manipulated NK cells from frozen peripheral blood mononuclear cells. By considering the expansion and cytotoxic activity of NK cells, KBM581 was identified as the optimal culture medium, which achieved the strongest cytotoxicity and greatest expansion within 2 weeks when supplemented with human AB serum and SR, respectively. Meanwhile, BaEV-enveloped lentivirus remains the preferred choice for viral transduction of NK cells. For gene editing, NK cells activated with feeder cells for 4–6 days strikes a balance between editing efficiency and cell viability. This research lays a solid foundation for the broader application of NK-cell immunotherapies. Despite these comprehensive findings, our study is limited by the small sample size. Future studies should incorporate a larger and more diverse donors to assess this expansion and gene modification platform.

## Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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## Author Contributions

Conception and design of the study: WM, LZ and LH. Acquisition of data: HLL, SWY, SJZ and TG. Analysis and interpretation of data: HLL, SWY, SJZ and TG. Drafting or revising the manuscript: HLL, SWY, WM, LZ and DJL. All authors have approved the final article.

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This paper is dedicated to the memory of the late Professor Jianfeng Zhou, who passed away unexpectedly on March 27, 2022. His profound commitment to mentoring and unwavering dedication to cellular immunotherapy continues to inspire.

## Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcyt.2024.10.006.

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