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ORIGINAL PAPER

Genetically engineered K562 cells augment NK cell cytotoxicity against acute myeloid leukemia and reduce dependency on IL-15

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Abstract

Acute myeloid leukemia (AML) is an aggressive malignancy with limited treatment options. Enhancing natural killer (NK) cell functionality through artificial antigen-presenting cells (aAPCs) represents a promising immunotherapeutic strategy. This study evaluates the potential of genetically modified K562 cells, expressing CD137L and CD86, to enhance NK cell-mediated cytotoxicity against AML cell lines (HL-60, KG-1, and THP-1). Lentiviral transduction was used to generate aAPCs, confirmed by PCR, RT-PCR, and flow cytometry. Cord NK cells and the NK-92 cell line were co-cultured with aAPCs, and their cytotoxicity against cell lines was assessed using 7-AAD staining. The ability of transduced K562 cells to substitute for interleukin-15 (IL-15) was also evaluated. These cells significantly enhanced NK cell-mediated cytotoxicity, with greater effects observed at higher effector-to-target (E:T) ratios. The aAPCs partially replaced IL-15 in activating cord blood NK cells but were ineffective for NK-92 cells. The aAPCs effectively enhance NK cell cytotoxicity and may reduce cytokine dependence in therapeutic applications. These findings highlight the potential of aAPCs to improve NK cell-based immunotherapies.

Keywords Artificial antigen-presenting cell · Natural killer cell · Acute myeloid leukemia · Cord Blood NK cells · NK-92 cells

Introduction

Acute myeloid leukemia (AML) is an aggressive blood cancer that causes disruptions in blood cell production and systemic complications [1]. Although current treatments such as chemotherapy, targeted therapy, and stem cell transplantation have advanced, relapse and resistance remain significant challenges. So, there is an urgent need for novel and innovative therapeutic approaches to improve treatment in AML patients [2].

Natural killer (NK) cells are a member of the innate immune system and have great cytotoxic abilities and a vital role in identifying and killing cancer cells. Unlike T cells, NK cells do not require antigen presentation via MHC molecules for activation, making them effective against cancer cells that evade the adaptive immune system by downregulating MHC-I expression. Various mechanisms are involved in the elimination of malignant cells by NK cells, such as death receptor signaling, cytokine release, tumor microenvironment regulation, antibody-dependent cellular cytotoxicity, and releasing cytotoxic granules [3, 4]. The NK cells can be categorized into primary NK cells, isolated from peripheral blood, umbilical cord blood, and bone marrow, and NK cell lines, in which the NK-92 cell line is the most used one. Cord blood-derived NK (CB-NK) cells exhibit high innate cytotoxicity, lower immunogenicity, and the potential for ex vivo expansion, making them promising for clinical applications. However, their initial cell numbers are limited, and they have a shorter lifespan in culture. In contrast, NK-92 cells, an established NK cell line, offer a high expansion rate, strong anti-tumor activity due to the absence of major

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inhibitory receptors (KIRs), and standardized use in research. Despite these benefits, NK-92 cells require irradiation before clinical use to prevent uncontrolled proliferation and depend on cytokine-enriched culture conditions for optimal function [5, 6].

Enhancing NK cell function through artificial antigen-presenting cells (aAPCs) can further improve their efficacy, offering promising strategies for cancer immunotherapy. These cells are engineered cell-based platforms designed to mimic the characteristics and functions of natural antigen-presenting cells [7]. K562 cells are highly suitable for generating aAPCs and can be genetically modified to express costimulatory molecules such as CD137L and CD86, enhancing their ability to activate immune cells [8]. CD137 ligand (CD-137L) or 4-1BB ligand (4-1BBL) is a transmembrane glycoprotein member of the tumor necrosis factor (TNF) superfamily. The interaction of CD137 or 4-1BB, expressed on the surface of activated T cells, natural killer NK cells, and some antigen-presenting cells (APCs), has a crucial role in immune responses for providing co-stimulatory signals that enhance T cell and NK cell activation, proliferation, and survival [9]. CD86, also known as B7-2, is also a transmembrane glycoprotein belonging to the B7 family [10]. It is expressed on the surface of APCs, including B cells, dendritic cells, and macrophages. CD86 plays a critical role in modulating T cell activation and immune responses through its interaction with CD28 and CTLA-4 receptors on T cells and also it plays an important role in activation and regulation of NK cells [11, 12].

Interleukin 15 (IL-15) is a cytokine that plays a crucial role in the development, survival, proliferation, and activation of immune cells including NK cells. It also inhibits NK cell apoptosis, by regulation of some proteins like Bim, Noxa, and Mcl-1 [13]. In addition, this cytokine increases NK cell cytotoxicity and production of other cytokines, such as interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) [14, 15]. However, in vitro experiments suggest that prolonged stimulation with soluble IL-15 complexes or monomeric IL-15 can induce NK cell hyporesponsiveness by directly affecting their activation and functionality [16].

Previous studies have primarily focused on the role of aAPCs in the isolation and expansion of primary NK cells and their impact on NK cytotoxicity [17–20]. However, few studies have directly compared the effects of aAPCs on the cytotoxicity of both CB-NK cells and the NK-92 cell line.

In this study, we aimed to generate and evaluate genetically modified K562 cells expressing 4-1BBL and CD86 as aAPCs to enhance the cytotoxic potential of CB-NK and NK-92 cells against AML cell lines. We assessed the ability of these aAPCs to promote NK cell-mediated lysis of HL-60, KG-1, and THP-1 cells and examined their potential as an alternative to interleukin-15 (IL-15) in NK cell activation. Our findings highlight the potential of engineered aAPCs to overcome current limitations in NK cell-based immunotherapy.

Materials and methods

Plasmid extraction

Plasmids including psPAX2 (Addgene Plasmid #12,260), pMD2.G (Addgene Plasmid #12,259) helper vectors and pCDH-EF1α−4-1BBL-P2 A-CD86-Puro (a gift from Dr. Jafar Kiani, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran) were extracted using the FavorPrep™ Plasmid DNA Extraction Kit (Favorgen Biotech Corp, Taiwan), following the manufacturer's protocol. Extracted plasmid DNA was then stored at −20°C for further use.

Cells and culture conditions

K562 cells (lymphoblast cells), HL-60 (acute promyelocytic leukemia), KG-1 (erythroleukemia) and THP-1 (acute monocytic leukemia) were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) while Dulbecco's modified Eagle's medium (DMEM) (Gibco) was used as HEK293 T (human embryonic kidney) culture media. All culture media were supplemented with 10% fetal bovine serum (Gibco), 100 µg/ml streptomycin, and 100 U/ml penicillin (Thermo Fisher Scientific, Inc Waltham, USA). These cells were purchased from the Iranian Biological Resource Center (IBRC) and incubated at 37 °C and 5% CO2.

Umbilical cord blood samples ($n = 3$) were obtained from healthy donors and collected in CPDA1 anticoagulant. These samples were procured from the cord blood bank of the Iranian Blood Transfusion Organization (IBTO) after obtaining written informed consent from the donors. For NK cell isolation, the Miltenyi Biotec NK Cell Isolation Kit was employed according to the manufacturer's protocol with minor modifications. Briefly, the umbilical cord blood was initially diluted 1:4 with PBS and carefully layered over Lymphoprep (Stemcell Technologies, Vancouver, Canada) for density gradient centrifugation (800 × g, 30 min, without brake, 20 °C) to isolate mononuclear cells. Following collection of the mononuclear cell layer and subsequent washing (300 × g, 10 min), the NK cell isolation procedure was initiated using the Miltenyi Biotec kit components. The mononuclear cells were incubated with the provided biotin-conjugated antibody cocktail (10 min, 4 °C) to specifically label non-NK cells, followed by adding MicroBead Cocktail (15 min, 4 °C). Magnetic separation was then performed by passing the cell suspension through a pre-washed MACS LS column placed in the Miltenyi MACS separator, allowing unlabeled NK cells to pass through while retaining labeled

non-NK cells. Finally, the NK cell-enriched fraction was collected, centrifuged (300 ×g, 10 min), and resuspended in the appropriate buffer.

The purity of extracted CB-NK cells was evaluated by analyzing the expression of CD3 and CD56 markers (BD Biosciences). The human NK-92 cell line was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, DSMZ numbers ACC 488). The CB-NK and NK-92 cells were cultured in RPMI medium (Gibco) enriched with 15% fetal bovine serum (FBS, Gibco), 2 mM glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Thermo Fisher Scientific, Inc), along with 500 U/ml interleukin-2 (IL-2) and 50 U/ml interleukin-15 (IL-15), (Miltenyi Biotec, Bergisch Gladbach, Germany). Hemocytometer and methylene blue staining techniques were performed for cell counting and assessing the viability of cells.

Lentivirus production

HEK293 T cells were cultured in T75 cm^2 flasks until reaching 70–80% confluency. Then, the media of cells was replaced with DMEM media completed with 2% FBS, without antibiotics, to starve cells for 2 h. Separately, the plasmids and polyethylenimine (PEI) 1 mg/ml (Sigma, St. Louis, MO) were mixed with DMEM media in a 1.5 ml microtube, and then 2 mixtures were combined at a specific ratio, incubated for 30 min at room temperature to prepare DNA-PEI complex which was added to starved HEK293 T cells. After 8 h, the medium was replaced with 10% FBS-DMEM media without antibiotics. After 48 h and then over the next 3 days, supernatants containing the secreted lentivirus particles were collected every 24 h, stored at 4 °C, and the cells were replenished with fresh medium to maintain optimal conditions for virus production. The collected viral supernatants were centrifuged at 2000 ×g 10 min and filtered through 0.45µm syringe filters to remove any cell debris. Virus enrichment was achieved by adding PEG8000 (Sigma) to a final concentration of 12% (w/v) and NaCl 5 M (5% final concentration) to the virus supernatant. The mixture was rotated overnight at 4 °C and subsequently centrifuged at 8000 rpm for 20 min to precipitate the virus particles. The pellet was resuspended in 3 ml media and stored at −80 °C until further use.

Puromycin Selection

Before transduction, the optimal dosage of puromycin for antibiotic selection was determinated. Briefly, K562 cells were seeded in appropriate plate wells, and treated by different dosages of puromycin (0.5, 1, 1.5, 2, 3 and 4 µg/ml) (Sigma). Every 2 days, the culture media was replaced with a fresh medium containing puromycin to ensure continuous antibiotic selection. Ultimately, a concentration of 2 µg/ml was selected as the optimum dose, which effectively removed all non-transduced K562 cells on day 6.

Transduction of K562 cells to make aAPCs

K562 cells were infected by prepared lentivirus containing CD137 and CD86 transgenes with 8 µg/ml of polybrene (Sigma) in two separate instances, 24 h apart. Following 72 h after the final lentivirus dose with the desired multiplicity (MOI), puromycin selection was conducted at a dose of 2 µg/ml (refer to Sect. "Puromycin Selection") to isolate and select successfully transduced K562 cells.

Approval tests for evaluation transduction efficiency

Two weeks after the last puromycin treatment, K562 cells were analyzed by PCR, RT-PCR (reverse transcription-PCR), and flow cytometry to evaluate the efficacy of transduction.

PCR and RT-PCR (Reverse Transcription-PCR)

The genomic DNA and total RNA of K562 cells were extracted using the DNsol mini kit (ROJE, Iran) and TRIzol reagent (RNX-Plus reagent, Sinaclon, Iran), respectively, according to the manufacturer's protocol. After the DNase I treatment, cDNA synthesis was done using a cDNA synthesis kit (Sinaclon, Iran). Briefly, total RNA (2 µg) and random hexamer primer (1 µl) were mixed, followed by Incubation at 65 °C for 5 min and chill on ice for 2 min. Then, cDNA synthesis mix, containing buffer (1 µl), M-MuLV reverse transcriptase (0.5 µl), RNase inhibitor (0.5 µl), dNTP Mix (2 µl), DEPC-treated water (5 µl), was added into RNA and random hexamer primer mixture. The reaction was performed in a SimpliAmp™ Thermal Cycler (Applied Biosystems, USA) with the following incubation steps: 25 °C for 5 min, 42 °C for 1 h, and finally 85 °C for 5 min to terminate the reaction. The synthetized cDNA directly was used in PCR, by the addition of 2µl of cDNA to the PCR reaction. The PCR reaction mixture was prepared in a final volume of 25 µl containing Taq DNA Polymerase Master Mix (Ampliqon, Denmark), cDNA, specific primers, and PCR-grade water. The amplification was then carried out using a SimpliAmp thermal cycler with the following program: initial denaturation at 95 °C for 4 min, followed by 33 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. Gel electrophoresis was used to evaluate the PCR results. Primer sequences specific for the CD86-P2A-41BBL sequence, and the *GAPDH* gene were used as follows:

Gene names	Primer sequence
CD86-P2A-41BBL	F: 5'-TCAGCCTGTCCGTGTCATTC-3' R: 5'-AGGTATCGCTCTTGTCGCAG-3'
GAPDH	F: 5'- TGAACGGGAAGCTCACTGG −3' R: 5'- TCCACCACCCTGTTGCTGTA-3'

Flow cytometry analysis of CD137L (4-1BBL) and CD86

K562 cells were resuspended and washed twice with PBS 1× (Thermo Fisher Scientific), then they were incubated with optimum concentration of PE anti-human CD137L antibody and FITC anti-human CD86 antibody (BioLegend, USA) for 15–20 min at 4 °C, protected from light. To remove the unbound antibodies, the cells were washed and centrifuged at 350 ×g for 5 min, and subsequently, the fluorescent signal of antibodies was read in appropriate flow cytometry (BD FACSLyric™ Flow Cytometry) channels.

Cytotoxicity assay

Before co-culturing, K562 cells were irradiated with 100 Gy of gamma radiation (Biobeam GM 8000, Germany). CB-NK cells and NK-92 cells were separately co-cultured with irradiated transduced and untransduced K562 cells. Then, for 2 weeks, they were used as feeders for both NK cells at a ratio of 20:1 (irradiated K562 to CB-NK and NK-92 cells). The medium was changed with fresh medium every 2–3 days. In the last 5 days, no new irradiated K562 cells were added (out of 14 days of co-culturing) to have a relatively pure population of NK cells at the end of the two weeks of culture for cytotoxicity testing. Flow cytometry results demonstrated that approximately 94% of the remaining cells were NK cells ($CD3^-$ $CD56^+$). Following this co-culture, the NK cells (both CB-NK and NK-92) were introduced to various target cell lines, including HL-60, KG-1, and THP-1, to evaluate their cytotoxic effects on these targets using flow cytometry and 7-AAD staining. In brief, the target cells were first labeled with carboxyfluorescein succinimidyl ester (CFSE) dye (Invitrogen, USA), and subsequently exposed individually to CB-NK and NK-92 cells at different effector-to-target (E:T) ratios of 1:1, 2:1, 5:1, and 10:1 for 4 h in a 96-well U-shaped plate at 37 °C and 5% CO2, and then the living cells were measured by 7-AAD staining by flow cytometry. The untreated target cells were labeled with CFSE and were used as a negative control to assess the viability of cells in the presence of CB-NK and NK-92 cells. The percentage of NK cell-mediated specific lysis of target cells was calculated using the following formula:

$$\%\ \text{specific lysis} = \left[\left(\%\ 7\text{-AAD}^+ \text{in experimental well} - \%\ 7\text{-AAD}^+ \text{in negative control well}\right) / \left(100 - \%\ 7\text{-AAD}^+ \text{in negative control well}\right)\right] \times 100$$

To examine the acting possibility of Transduced K562 cells, as feeder cells, substitution for IL-15 in the culture medium, CB-NK and NK-92 cells were co-cultured with IL-2 (500 U/ml) and feeder cells for 14 days under the same conditions described earlier. As a control, NK cells were cultured with IL-2 (500 U/ml) and IL-15 (50 U/ml) in the absence of feeder cells to compare the effects on NK cell growth and activation. Different E:T ratios of 5:1, 8:1, 12:1, 15:1 and 20:1 were examined after 4 h incubation at 37 °C and 5% CO2 in a 96-well U-shaped plate. Finally, the 7-AAD staining was performed to measure the living cells. All experiments were done in triplicate.

Flow cytometric assessment of cell viability

Firstly, cells were washed with PBS to eliminate any residual media or debris after 4 h of incubation of target cells with NK cells. Next, the 7-AAD solution was added to the cells at a final concentration specified by the manufacturer. The cells were incubated for 15–30 min at 4 °C in a dark place. The target cell population was determined and gated as $CFSE^+$ cells, then the necrotic or apoptotic 7-AAD^+ cells were measured in the gated target population.

Statistical analysis

Experimental results are expressed as the mean value with standard deviation (SD) using GraphPad PRISM 8 software (GraphPad Software). T-test, one-way and two-way ANOVA tests were used to determine statistical significance. A P-value less than 0.05 was considered statistically significant.

Results

Purity of CB-NK cells

Flow cytometric analysis of CD3 and CD56 surface markers was performed to examine the purity of CB-NK cells. As shown in Fig. 1, approximately 97% of the cells were $CD3^-$ $CD56^+$, confirming the high purity of the NK cell population extracted from umbilical cord blood. The non-specific binding was not observed when cells were stained by isotype control antibodies, indicating the specificity of antigen-antibodies bindings.

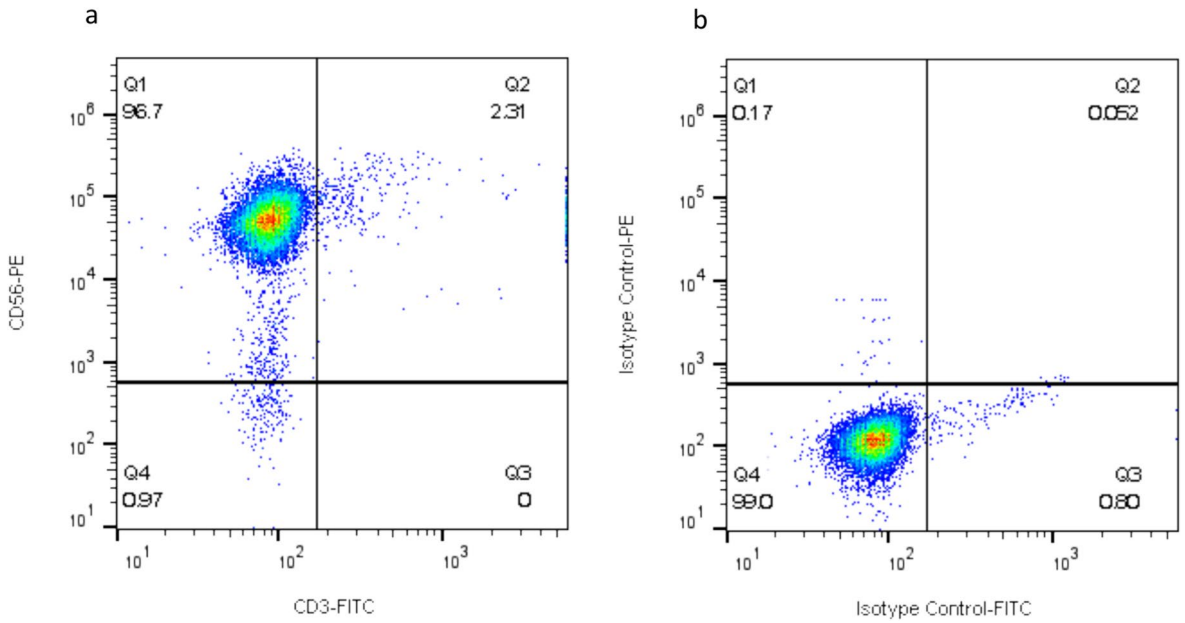

Fig. 1 CB-NK cell markers analysis. **a** Nearly 97% of the cells were $CD3^{-}$ $CD56^{+}$, confirming their high purity. **b** The isotype control analysis validated the specific binding of antibodies

Puromycin selection

To enrich the transduced cell population, a Puromycin selection process was employed and cells were treated with 2 µg/ml of puromycin, as the optimal dosage of antibiotic in which all untransduced cells were not capable of surviving after treatment (Fig. 2).

Transduction of K562 cells

K562 cells were genetically modified using lentiviral vectors encoding 4-1BBL (CD137L) and CD86, to finally generate 4-1BBL-CD86-K562 cells as artificial antigen-presenting cells (aAPCs). Two weeks after the last dosage of puromycin, the efficiency of K562 transduction was assessed

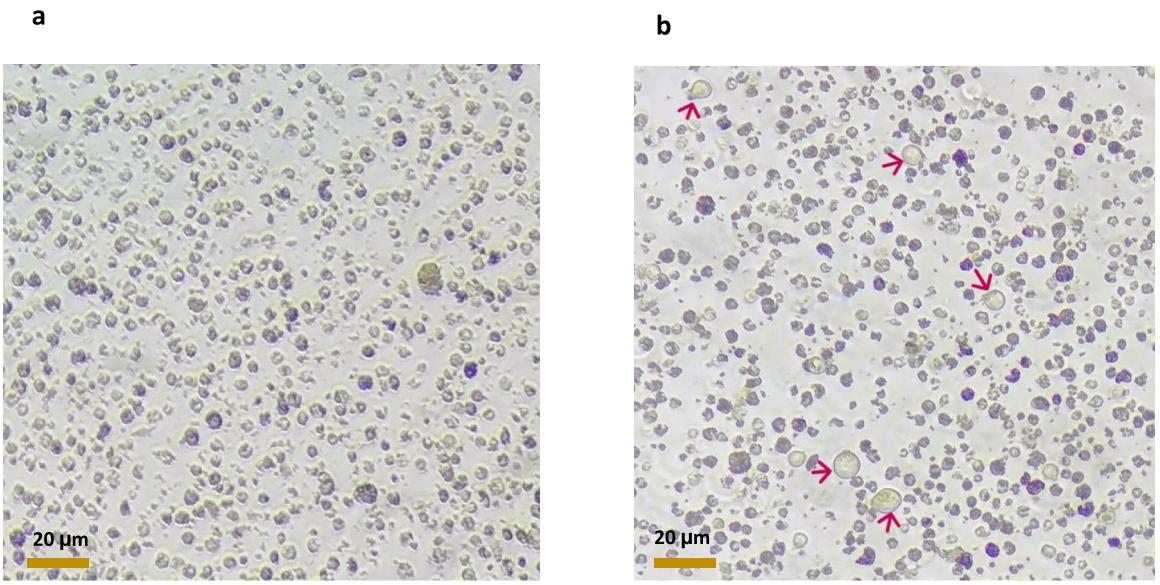

Fig. 2 Puromycin selection of K562 cells with 2 µg/ml puromycin for 6 days. **a** All untransduced cells (control) died, and no living cells were observed. **b** Red marks indicate living cells, demonstrating that cells were successfully transduced with lentivirus containing a puromycin resistance gene

through PCR, RT-PCR, and flow cytometric analysis as follows:

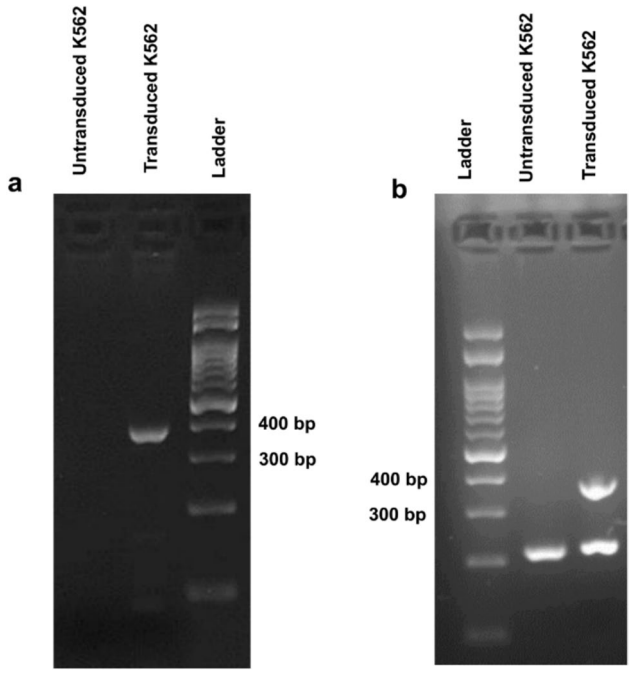

Fig. 3 Agarose (2%) gel electrophoresis of amplification products. **a** Amplification of 381 nucleotides confirmed integration of transgene (CD86-P2 A-CD137L) into K562 cells genome. **b** The RT-PCR analysis of extracted RNA from these cells showed transgene expression only in transduced K562 cells. GAPDH was used as a housekeeping gene

PCR and RT-PCR

As demonstrated in Fig. 3, PCR reactions using genomic DNA and cDNA templates using specific primers for transgene resulted in the detection of an approximately 381-nucleotide amplicon, confirming the successful integration (Fig. 3a) and expression of transgene (CD86-P2 A-CD137L) (Fig. 3b) into transduced K562 cells.

Flow cytometry

Flow cytometry data demonstrated successful transduction where almost 43% of cells expressed CD137L and CD86 antigens in comparison to untransduced ones (control) (Fig. 4).

Cytotoxic activity of CB-NK and NK-92 cells against acute myeloid leukemia (AML) cell lines

CB-NK and NK-92 cells were separately co-cultured with irradiated transduced (TK562) and intact K562 cells (as a feeder) for 2 weeks (with 500 U/ml IL-2 and 50 U/ml IL-15), and then their cytotoxicity properties were examined against HL-60, KG-1 and THP-1 cells (AML cell lines). For more detail, refer to the 2.7 and 2.8 sections. Figure 5 shows the impact of feeder cells (TK562 and K562) and the absence of feeder cells on NK cell cytotoxicity at an E:T ratio of 5:1. Further details are provided below. In addition, Fig. 6 demonstrates the effect of various E:T ratios (1:1, 2:1, 5:1, and 10:1) on NK cell cytotoxicity. The results of HL-60,

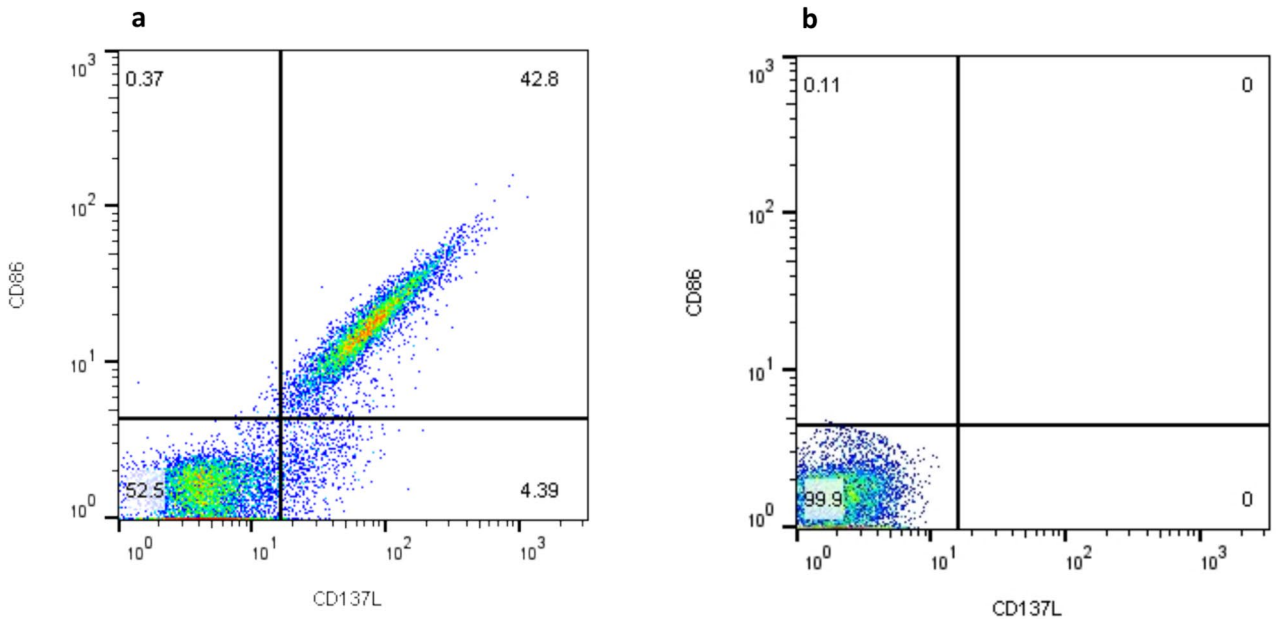

Fig. 4 Transduction efficacy of K562 cells to produce aAPCs. **a** 43% of cells expressed CD137L and CD86, indicating the transduction yield and the transduced K562 cell population. **b** Almost all unmodified cells (control) did not express CD137L and CD86

KG-1, and THP-1 cells lysis obtained from 7-AAD staining are presented in Fig. 5 and 6. Also, Fig. 6 a-c presents flow cytometry histograms depicting the percentage of 7-AAD$^+$ cells within the target cell populations.

HL-60

At an effector-target ratio of 5:1, virus-transduced cells expressing 4-1BBL and CD86 (aAPCs), showed a positive effect on the killing efficiency of CB-NK cells (Fig. 5a). CB-NK cells co-cultured with TK562 demonstrated a higher lysis rate against HL-60 cells, compared to the CB-NK cells cultured with untransduced K562 cells ($p < 0.0001$) or in the absence of feeder cells ($p < 0.0001$). This suggests that TK562 cells can serve as an effective feeder for CB-NK cells when targeting HL-60 cells. While, the untransduced K562 cells appear to be an ineffective feeder, as the lysis rate of target cells remains similar when CB-NK cells are either co-cultured with K562 cells or in the absence of feeder cells ($p = 0.8$).

The same experiment was conducted using NK-92 cells, and a positive influence of TK562 cells on the lysis rate of HL-60 cells was observed. NK-92 cells co-cultured with TK562 cells lysed 30% of HL-60 cells, whereas cultured with K562 ($p = 0.0001$) cells or without feeder cells ($p < 0.0001$) lysed approximately 22% of the target cells. These findings indicate that TK562 cells can enhance the cytotoxicity of NK-92 cells against HL-60 cells, while the untransduced state does not exhibit a significant impact on the lysis rate.

Comparing the two NK cell types, NK-92 cells appear to have higher cytotoxic potential than CB-NK cells, while no significant difference was observed between NK-92 and CB-NK cells when cultured with TK562 cells. This suggests that NK-92 cells possess a higher inherent lethality than CB-NK cells against HL-60 cells.

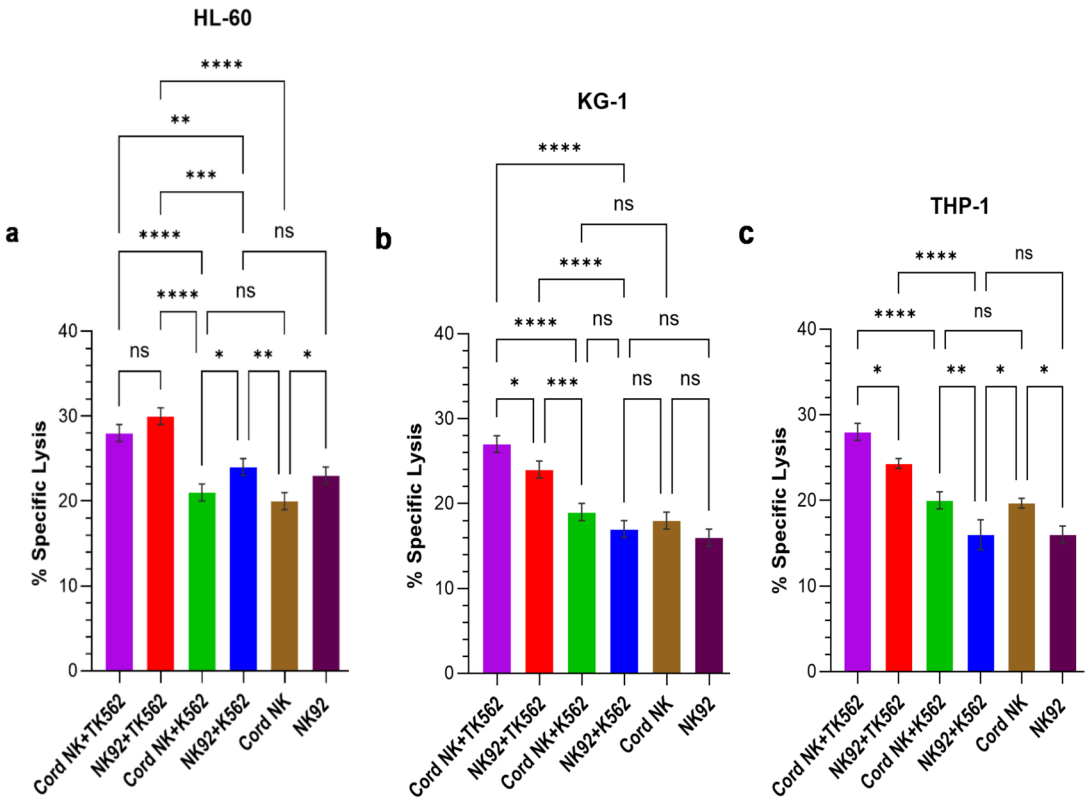

Fig. 5 CB-NK and NK-92 cells were co-cultured with TK562 (transduced K562) and K562 (untransduced K562) cells (at E:T ratio of 5:1) in the presence of IL-2 and IL-15, and their cytotoxic properties were evaluated against target cells. **a** Co-culturing CB-NK and NK-92 cells with TK562 cells resulted in an enhanced cytotoxic effect against HL-60 cells while those cultured with K562 cells or without feeder cells showed lower toxicity. Moreover, a comparison between the two NK cell types suggests that NK 92 cells inherently possess a higher cytotoxic potential than CB-NK cells against HL-60 cells. **b** TK562 cells significantly enhance the cytotoxicity of both CB-NK and NK-92 cells against KG-1 cells, exhibiting similar lysis rates to those observed against HL-60 cells. **c** In the case of THP-1, the presence of aAPCs significantly increased the cytotoxicity of NK-92 and CB-NK. Data represent mean ± SD from three independent experiments. Statistical significance was determined by one-way ANOVA: ns (not significant), $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

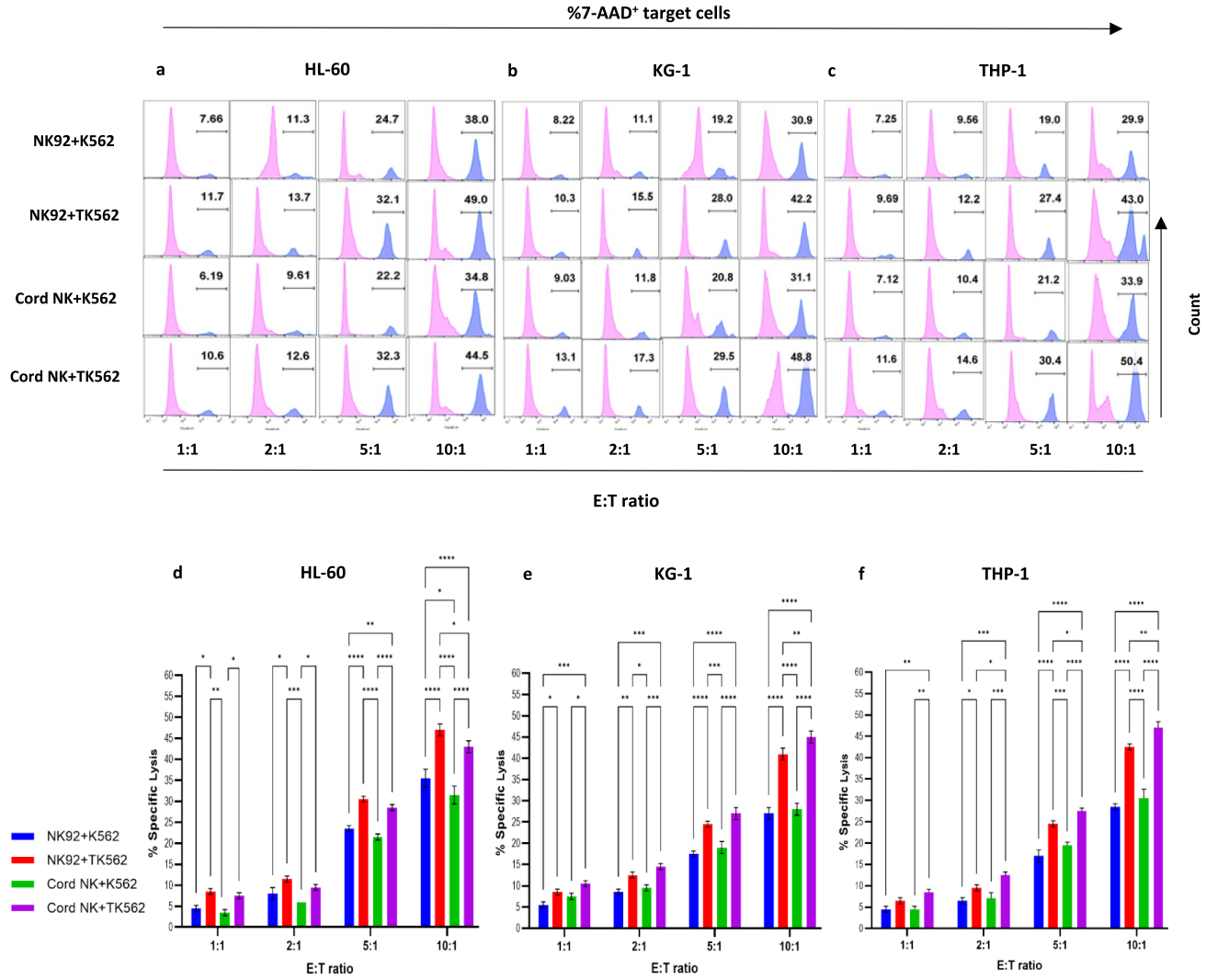

Fig. 6 Co-culture of NK cells with TK562 and K562 cells at various effector-to-target (E:T) ratios. Flow cytometry histograms **a**–**c** depict target cells (HL-60, KG-1, THP-1) labeled with CFSE to distinguish them from NK cells (gating steps not shown). The percentages of apoptotic and necrotic cells were then quantified using the fluorescent dye 7-AAD. Higher effector-to-target ratios correlated with increased cytotoxicity, as evidenced by a rise in 7-AAD$^+$ cells. Panels d-f display the mean ± standard deviation of target cell-specific lysis across three independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's multiple comparisons test. In cases where significance was not shown, the results indicated no statistically significant differences. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

KG-1

In a co-culture of CB-NK cells with TK562 cells against KG-1, the lysis rate was found to be similar to that observed for HL-60 cells (Fig. 5b). When examining NK-92 cells, a positive effect of TK562 cells on the lysis rate of target cells was observed and their cytotoxicity was more than those in HL-60. NK-92 cells co-cultured with TK562 cells lysed 24% of HL-60 cells, whereas NK-92 cells cultured with untransduced K562 ($p < 0.0001$) cells or without feeder cells ($p < 0.0001$) demonstrated a lysis rate of approximately 16% against the target cells. The untransduced state of K562 cells does not exhibit a significant influence on the cytotoxicity of NK cells, however, TK562 cells may be considered a suitable feeder to improve the cytotoxic functions of NK-92 and CB-NK cells in targeting KG-1 cells.

THP-1

Figure 5c demonstrates the cytotoxic effects of CB-NK and NK-92 cells against THP-1 cells. The results showed that co-culture with TK562 cells significantly increased the cytotoxicity of both NK cell types on THP-1 cells. Consistent

with previous findings, intact K562 cells did not show a significant impact on their cytotoxicity. Moreover, CB-NK cells exhibit significantly higher cytotoxicity against THP-1 target cells compared to NK-92 cells, in the presence or absence of feeder cells (TK562 and untransduced K562).

These findings further support the potential of aAPCs as an effective feeder to improve the cytotoxic functions of both CB-NK and NK-92 cells when targeting HL-60, KG-1, and THP-1 cells (AML cell lines).

Figure 6 presents the percentage results for various E:T ratios, demonstrating that increasing the ratio of NK cells to target cells in different culture conditions leads to enhanced cytotoxicity. Notably, the presence of aAPCs as feeders results in a significantly greater increase in cytotoxicity, suggesting that aAPCs play a crucial role in promoting NK cell activation and cytotoxic function.

aAPCs as potential substitutes for IL-15

To examine the possibility of using aAPCs as an alternative to IL-15 when used as a feeder, CB-NK and NK-92 cytotoxicity against target cells in the presence and absence of IL-15 was examined (Fig. 7). IL-2 (500 U/ml) was used at a constant concentration in all conditions. Control samples were supplemented with IL-2 (500 U/ml) and IL-15 (50 U/ml) to facilitate comparisons between the effect of IL-2, IL-15, and feeder cells on NK toxicity. The experiment is explained in Sect. "Cytotoxicity assay" in detail. The data presented in Fig. 7 represents the average percentage of 7-AAD$^+$ cells across HL-60, KG-1, and THP-1 cell lines. At effector-to-target ratios of 5:1 and 10:1, CB-NK cells treated with IL-2 and IL-15 showed a significantly higher level of cytotoxicity compared to conditions where TK562 cells (aAPCs) served as a feeder (without IL-15), with p-values of 0.02 and 0.009, respectively (Fig. 7a). By increasing the effector-to-target ratio, the cytotoxicity of CB-NK cells in the condition where aAPCs was used as a feeder became similar to that in conditions without a feeder but supplemented with IL-15. At ratios of 15:1 and 20:1, aAPCs were able to substitute for IL-15, as no significant difference was observed between the groups. These findings indicate that aAPCs may serve as a suitable alternative to IL-15 in promoting the cytotoxic functions of CB-NK cells under specific conditions and E:T ratios.

In contrast to the findings with CB-NK cells, the removal of IL-15 had a significant negative impact on the cytotoxicity of NK-92 cells. The presence of aAPCs as feeder was unable to replace IL-15 for NK-92 cells at any effector-to-target ratio (Fig. 7b), indicating that aAPCs could not serve as an alternative to IL-15 for maintaining optimal NK-92 cell cytotoxic functions in these experimental conditions.

Discussion

Utilizing aAPCs to improve NK cell function shows potential for advancing cancer immunotherapy. Past research has primarily focused on the application of aAPCs in the

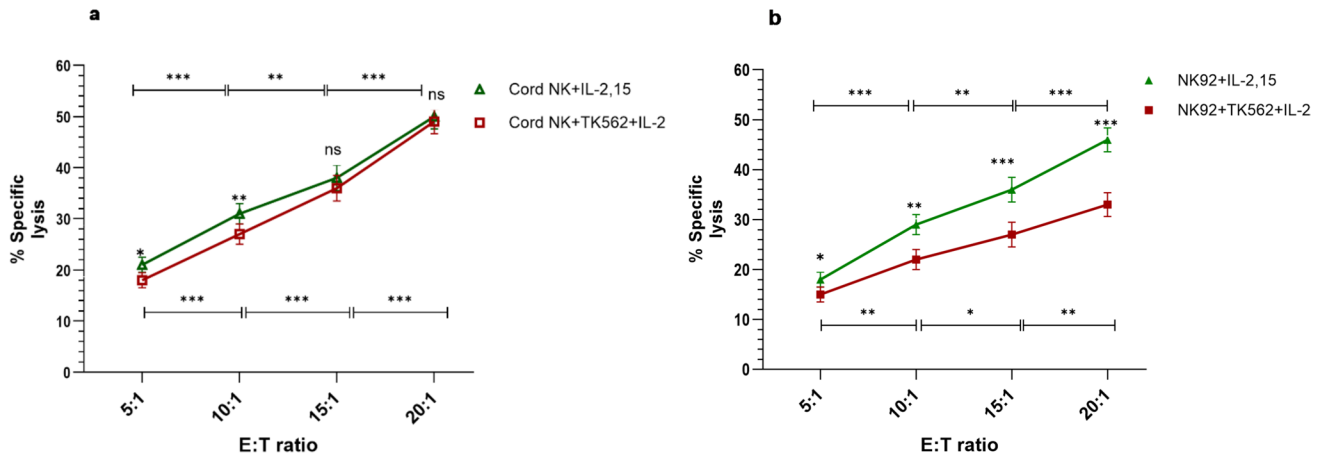

Fig. 7 Evaluating the potential of aAPCs as IL-15 substitutes for enhancing CB-NK and NK-92 cells cytotoxicity. The percent lysis of target cells represents the average percentage of 7-AAD$^+$ cells across HL-60, KG-1, and THP-1 cell lines. **a** Examination of TK562 cells (aAPCs) as IL-15 substitutes for CB-NK cells against target cells revealed that these cells effectively enhanced CB-NK cell cytotoxicity at higher effector-to-target ratios, potentially serving as an alternative to IL-15. **b** aAPCs failed to substitute for IL-15 in NK-92 cells, as their removal significantly decreased NK-92 cell cytotoxicity. Data are presented as mean ± SD from three independent experiments. Statistical significance was determined using Student's t-test, with significance thresholds defined as: ns (not significant), $P \geq 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

isolation and expansion of primary NK cells and their influence on NK cytotoxicity. However, less attention has been given to comparing the effects of aAPCs on the cytotoxic potential of both CB-NK cells and the NK-92 cell line. K562 cells are commonly used for cell-based aAPCs due to their particular properties. For instance, the lack of HLA expression prevents allogeneic responses and KIRs activation, making them ideal for expanding NK cells without triggering undesired immune reactions. Also, K562 cells can stably express a wide range of molecules on their surface, making them suitable for gene transduction and have adhesion molecules that enhance interactions between cells and aAPCs [21, 22]. Co-culturing of clinical-grade NK cells with K562 cells as aAPCs enhanced their cytokine secretion, expansion, and function, indicating potential application for improving NK cell-based cancer immunotherapy in patients with advanced malignancies [23]. K562-based aAPCs expressing CD86, CD137L, and membrane-bound IL-15 boosted peripheral blood-derived NK cell activation, survival, and proliferation. This optimized system achieves high-purity NK cell expansion, supporting applications in immunotherapy and CAR-NK development [24].

K562 cells expressing CD80 alone or 4-1BBL alone can promote prolonged proliferation of CD8 + T cells. Additionally, other studies have shown that the inclusion of 4-1BBL helps preserve TCR diversity in T cells and reduces apoptosis in CD8 + T cells. Notably, the co-expression of CD80 and 4-1BBL on aAPCs has been shown to be more effective than using APCs expressing only one of these agonist molecules [22].

CD137 and CD137L interaction result in enhancing the anti-tumor activity of immune cells, particularly NK cells. This interaction stimulates NK cells to produce cytokines like IFN-γ, which can help create an anti-tumor environment by recruiting and activating other immune cells, promoting cancer cell death, and inhibiting tumor growth [25–27]. The efficacy of aAPCs in activating T cells through CD28 and CD137 costimulatory was investigated. The aAPCs were synthesized using the addition of anti-CD137 and anti-CD28 antibodies to microbeads coated with MHC-peptide and anti-CD28. This approach successfully generated a high quantity of functional cytotoxic T lymphocytes with an effector memory subtype ($CD45RA^{-}$ $CCR7^{-}$) [28]. The aAPCs expressing membrane-bound IL-15 and CD137L not only enhanced NK cells expansion, but also surpassed using cytokines. The expanded NK cells showed increased cytotoxicity and could effectively target leukemia cells in vitro and in murine models [29]. Moreover, the data of two clinical trials using IL-15/CD137L activated and expanded NK cells in childhood leukemia/lymphoma patients revealed the safety of infusion of these cells in combination with chemotherapy [30].

CD86 expression on tumor cells has been associated with increased susceptibility to NK cell-mediated lysis. This could be due to the activation of NK cells through the interaction with CD28 or other yet unidentified mechanisms [31]. NK cells are involved in xenograft rejection through Galα1,3-Gal and CD86-dependent mechanisms. Porcine cells expressing CD86 are highly susceptible, but genetic modification to block CD86 significantly reduced NK cytotoxicity [32]. CD86 can also act as triggering molecules for NK cell-mediated cytotoxicity. The expression of human CD86 or CD80 in murine B16.F1 melanoma cells led to increased lysis susceptibility by human NK cell lines, but blocking the transfected gene products with specific monoclonal antibodies reduced the lysis rate [33]. CD86 may also promote NK cell survival and activation, possibly by inducing the production of cytokines and chemokines that influence NK cell function [34].

Maus et al. developed aAPCs cells, artificially manipulated cells designed to mimic the properties of natural APCs, to activate and quickly expand cytotoxic T cells. The K562 cells were engineered to stably express anti-CD28 and CD137L to interact with CD28 and CD137 receptors on the surface of cytotoxic T cells, leading to their activation and expansion [35]. K562 cells expressing CD137L and/or CD80 have been used to efficiently expand T cells and maintain CD28 expression on them [36]. aAPCs cells expressing CD137L and IL-15Rα stimulated activation of NK cells, resulting in significant upregulation of NKG2D, TRAIL, and natural cytotoxicity receptors (NCRs) [20].

IL-15 is essential for the development, survival, activation, and proliferation of NK cells. It exists in two forms: as a monomer (sIL-15) or as a complex with IL-15Rα (sIL-15/IL-15Rα). However, prolonged exposure to these forms can lead to NK cell hypo-responsiveness, limiting their effectiveness [37, 38]. The overproduction of sIL-15/IL-15Rα may also contribute to immune escape in cancer [38]. Moreover, high production costs, side effects, and its short half-life requiring repeated administration also restricted their use in clinical applications. To overcome these issues, researchers are investigating alternative approaches [39, 40]. The ability of aAPCs to substitute for IL-15 was examined in this study. While genetically engineering K562 cells expressing 4-1BBL and CD86 could replace IL-15 in enhancing the cytotoxicity of CB-NK cells at a high E:T ratio of 1:20, they were unable to substitute IL-15 for NK-92 cells under the same conditions. This suggests that while aAPCs can provide essential costimulatory signals, they may not fully recapitulate the cytokine milieu required for optimal NK-92 cell activity.

These results show the potential application of using genetically modified K562 cells as a support system,

feeder, or aAPCs could improve the cytotoxicity of NK cells *invitro*, leading to be applied for immunotherapy. Overall, the results showed that the cytotoxicity of NK cells varies depending on the target cells tested. Also, the findings indicate that aAPCs may serve as a suitable alternative to IL-15 in promoting the cytotoxic functions of CB-NK cells under specific conditions and E:T ratios.

Conclusion

Our study demonstrates that engineered K562 cells expressing 4-1BBL and CD86 (aAPCs) significantly enhanced the cytotoxic activity of both cord blood-derived NK (CB-NK) cells and NK-92 cells against AML cell lines (HL-60, KG-1, and THP-1). These findings suggest aAPCs may deliver co-stimulatory signals for NK cell activation in vitro. Notably, aAPCs partially compensated for IL-15 in CB-NK cell cultures, indicating a possible reduction in cytokine dependency; however, this effect was not observed in NK-92 cells, highlighting cell-type-specific limitations. While these findings support further exploration of 4-1BBL/CD86-expressing K562 cells for NK cell-based immunotherapy in AML, additional studies are needed to validate their efficacy in primary patient-derived models and to assess long-term functional persistence. The study's scope was limited to in vitro assays, and the translational relevance remains to be tested in preclinical or clinical settings.

Author contributions MS conceptualized the project idea and designed the study. SSA and JK performed the experiments and analysed the data. The study was supervised by MS and MSZ. The initial draft of the manuscript was prepared by SSA, while the final draft was written and revised by SSA and MSZ. All authors have reviewed and approved the final version of the manuscript for publication. This work has not been previously submitted, either in whole or in part, as a research article to any journal. The article has received approval from all listed authors.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflicts of interest The authors declare they have no financial interests. The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Tarbiat Modares University, Tehran, Iran (Date 2024.01.06/No. IR.MODARES.REC.1402.179).

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