

Research article

More balance toward activating receptors and cytotoxic activity of NK cells ex vivo differentiated from human umbilical cord blood-derived CD34⁺ stem cells in comparison with peripheral blood NK cells

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ABSTRACT

Adoptive immunotherapies that use functional NK cells depend on the availability of sufficient numbers of these cells. We expanded umbilical cord blood (UCB)-CD34⁺ HSCs for 2 weeks and then differentiated them into NK cells and compared their function to peripheral blood (PB) NK cells. We assessed NKG2D, NKG2A, NKp30, NKp44, NKp46, and the expression of CD107a, CD57, CD69, FasL, PD-1, and IFN- γ level in two groups after co-culture with K562 cell line. We found that UCB-CD34⁺-derived NK cells express significantly more NKG2D, NKp44, and NKp46 receptors than PB NK cells. PB NK cells expressed significantly higher NKG2A and CD57 than UCB-CD34⁺-derived NK cells. In addition, UCB-CD34⁺-derived NK cells significantly expressed CD107a more than PB NK cells. Based on our findings, UCB-CD34⁺ cells can be a potentially advantageous source with strong cytotoxic function to produce allogeneic NK cells for adoptive cancer immunotherapy.

1. Introduction

Predominant innate lymphocytes known as natural killer (NK) cells play an important role in defense against viral infections and cancer immunosurveillance. Unlike T cells and B cells, NK cells do not require prior sensitization, recognition of specific antigens, or clonal expansion. The natural cytotoxicity of these cells can be triggered quickly and is regulated by a complex balance of signals coming from germline-encoded activating and inhibitory receptors on their surface [1,2]. Surface major histocompatibility complex (MHC) class I molecules in healthy cells protect them from NK-mediated lysis by binding to inhibitory receptors on NK cells and blocking their effector functions. The opposite occurs in transformed cells, where MHC class I molecules are downregulated and stress-induced ligands such as MICA/B (MHC class I polypeptide-related sequence A/B) are upregulated, allowing for their recognition by NK cells and subsequent lysis [3,4]. Different allogeneic NK cell adoptive transfer approaches are being used for cancer treatment because of these unique properties [5].

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A significant obstacle to the clinical efficacy of NK cell immunotherapy has been the difficulty in obtaining enough cells for adoptive transfer since NK cells represent a small percentage of peripheral white blood cells, expand poorly *ex vivo*, and have short life spans. In order to achieve a successful NK-cell-based therapy, it is crucial to get enough effector cells with high purity and antitumor activity. Reliable techniques for manufacturing huge numbers of highly cytotoxic NK cells would be extremely beneficial for NK cell-based therapeutics. It has been reported that different approaches can be used to expand NK cells *ex vivo* on a large scale [6–8].

The development of CD56⁺ NK cells has been achieved by differentiation of different stem cell types, including hematopoietic stem and progenitor cells (HSCs) from bone marrow and umbilical cord blood (UCB), human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPSCs) [9–12]. Not only is UCB an attractive source of HSC for allogeneic stem cell transplantation (SCT), but it can also be used to create NK cells and other therapeutic products [10,13,14]. A number of research projects have documented the efficient production of UCB-CD34⁺-derived NK cells that exhibit improved effector functions and cytolytic activity against tumor cells *in vitro* and *in vivo* [10,12,15,16].

Here, we used the method of Oberoi et al. [17] based on sequential exposure to different cytokine cocktails for *ex vivo* differentiation of UCB-CD34⁺ cells into functional NK cells. The percentage of *ex vivo* differentiated CD3⁺CD56⁺ NK cells was measured. In addition, the surface expression of NK-cell-associated activating and inhibitory receptors, as well as cytotoxicity and cytokine production against a tumor cell line, was examined to characterize the resultant CD3⁺CD56⁺ NK cell product. Finally, we aimed to evaluate the phenotype, cytotoxic activity, and cytokine production of these cells in comparison to the phenotype, cytotoxic activity, and cytokine production of NK cells isolated from peripheral blood (PB) as one of the main sources of donor NK cells. Considering that UCB-CD34⁺ cells have a relatively high proliferation capacity, they are considered a suitable source for differentiation into NK cells. It is therefore possible to use differentiated cells for NK therapy and genetic manipulation of the resulting NK cells if they possess acceptable activating phenotypes against target cells.

2. Materials and methods

2.1. Cell line

K562 cells, an MHC-negative human erythroleukemia cell line was purchased from Pasteur Institute of Iran (Tehran, Iran) and maintained in Roswell Park Memorial Institute (RPMI) 1640 and supplemented with 10 % fetal bovine serum (FBS), GlutaMax (1X), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco) at 37 °C and 5 % CO₂.

2.2. Donor selection criteria

After receiving written informed consent with regard to scientific use, UCB units were taken at birth following a normal full-term cesarean delivery from the cord blood bank of the Isfahan Royan Institute (Isfahan, Iran). The mother was diagnosed healthy before collection and was not infected with HIV, HBV, HPV, or HCV. Their ages ranged from 22 to 34 years. Samples were collected in 250 ml sterile bags, containing 35 ml of anticoagulant Citrate Phosphate Dextrose Adenine Solution, USP (CPDA-1). A total of 3 human UCB samples were processed. After being collected, UCB samples were kept at room temperature and processed within 4 h after collection.

2.3. Isolation of UCB-CD34⁺ cells

UCB-CD34⁺ cells were isolated using a RosetteSep CD34 pre-enrichment cocktail followed by CD34⁺ selection using an EasySep Human Cord Blood CD34 Positive Selection Kit II (17896, Stemcell Technologies, Vancouver, BC, Canada). To confirm purity, isolated cells were stained in phosphate-buffered saline (PBS) supplemented with 2 % FBS at 4 °C for 25 min with Phycoerythrin (PE)-conjugated anti-human CD34 antibody (Stemcell Technologies). Stained cells were washed once with PBS supplemented with 2 % FBS and cell purity was analyzed by flow cytometry. Finally, the obtained UCB-CD34⁺ cells were used directly for the NK cell generation.

2.4. Expansion and differentiation of UCB-CD34⁺ cells into NK cells

UCB-CD34⁺ cells were used in a two-step protocol, consisting of 2 weeks of proliferation and 2 weeks of differentiation. Purified CD34⁺ cells were expanded for 14 days in 25 cm² culture flasks in CellGenix® GMP SCGM (Serum-free Stem Cell Growth Medium) (Cell Genix, Freiburg, Germany) supplemented with 10 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and cytokine cocktail 1 including recombinant human stem cell growth factor (SCF; 30 ng/ml), recombinant human fms-like tyrosine kinase 3 ligand (FLT3L; 50 ng/ml), recombinant human IL-6 (25 ng/ml), and recombinant human thrombopoietin (TPO; 25 ng/ml) (all from BioLegend, San Diego, CA, USA). For generation and development of NK cells, from day 15 to day 28, the expanded CD34⁺ cells were transferred to a differentiation medium containing NK MACS basal medium with 1 % NK MACS supplement (Miltenyi Biotec, Bergisch Gladbach, Germany), 10 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and cytokine cocktail 2 including SCF (30 ng/ml), FLT3L (50 ng/ml), recombinant human IL-7 (50 ng/ml), recombinant human insulin-like growth factor 1 (IGF-1; 100 ng/ml), recombinant human IL-15 (50 ng/ml), and recombinant human IL-2 (500 IU/ml) (all from BioLegend). During the expansion and differentiation phases, the half-medium changed every 2–3 days and cytokines were added with the same initial concentration. At day 28, cells were collected and the percentage of CD3⁺CD56⁺ NK cells was measured by flow cytometry. Using FSC to SSC plots, the debris population was removed and target cells were gated. The percentage of living cells was checked by 7-Aminoactinomycin D (7-AAD) staining, which was usually above 85 %. Then cells were used to stain with anti-CD3-FITC, anti-CD16-PE, and anti-CD56-PerCP or PE-Cy5 (BioLegend). The NK

cells were determined to be those that were CD3⁺CD56⁺. Additionally, appropriate isotope controls were used to eliminate non-specific binding. If differentiation was low, it continued until day 35.

2.5. Isolation and expansion of CD3⁺CD56⁺ NK cells from PB

PB samples were taken from three healthy people. Participants' written informed consent was obtained before obtaining blood samples. Healthy young donor's peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using 1.077 g/ml Ficoll-Histopaque (Sigma, St. Louis, MO, USA) and washed twice with PBS. CD56 cell fraction from PBMCs was obtained by magnetic negative selection, with a human NK cell isolation kit (MACS-Miltenyi Biotec), according to the manufacturer's instructions. Flow cytometry assessments of CD56⁺ and CD3⁺ markers were used to characterize the purity of isolated NK cells. CD56⁺ lymphocytes were cultured in NK cell media supplemented with 10 % FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, IL-15 (50 ng/ml), and IL-2 (500 IU/ml) for one week. After 7 days, NK colonies were detected and every three days, the culture medium was replaced with fresh IL-2 and IL-15-containing medium.

2.6. Phenotyping of UCB-CD34⁺-derived NK and PB NK cells

We co-cultured UCB-CD34⁺-derived NK and PB NK cells with K562 cells at 1:1 and 2:1 E:T ratios in a 96-well U-bottom plate for 4 h. We also considered the culture of UCB-CD34⁺-derived NK cells and PB NK cells alone as a negative control group. For direct surface staining of UCB-CD34⁺-derived NK and PB NK cells, the following antibodies were used according to the manufacturer's instructions: CD69-FITC, NKG2D-PE, NKG2A-APC, NKp30-APC, NKp44-PE, NKp46-PE, CD57-FITC, FasL-PE, and PD-1-APC (all from BioLegend). Stained cells were washed once with PBS supplemented with 2 % FBS and analyzed by flow cytometry. Using FSC to SSC plots, the debris population was removed and target cells were gated. The percentage of living cells was checked by 7-Aminoactinomycin D (7-AAD) staining, which was usually above 90 %. Additionally, appropriate isotope controls were used to eliminate non-specific binding. Data were acquired using a FACS Calibur cytometer (BD Biosciences). In the analysis, 30,000 cells were scored. All the treatments were performed in triplicates. The mean values of three replicates for each sample were used in the analysis.

2.7. Degranulation of UCB-CD34⁺-derived NK and PB NK cells

Cell surface expression of CD107a (LAMP-1) was used to measure the degree of UCB-CD34⁺-derived NK and PB NK cell degranulation during co-culture with K562 cells at 1:1 and 2:1 E:T ratios in a 96-well U-bottom plate. NK cells stimulated with PMA (50 ng/ml) plus ionomycin (1 µg/ml) served as positive control or left untreated as negative control. Anti-human CD107a-FITC antibody (eBioscience™, San Diego, CA) was added at the start of the co-culture, and brefeldin A (1 ng/ml, BD Biosciences) was added 1 h later. Cells were collected after 4 h of incubation at 37 °C, washed, and stained for PE-Cy5-labeled murine monoclonal antibody to human CD56 cell surface marker, and the proportion of CD107a⁺ cells was assessed. All the treatments were performed in triplicates. We also considered the culture of UCB-CD34⁺-derived NK cells and PB NK cells alone as a negative control group. The mean values of three replicates for each sample were used in the analysis.

2.8. Apoptosis analysis by flow cytometry

Apoptosis induction by UCB-CD34⁺-derived NK and PB NK cells was evaluated by using the BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. Effector cells were incubated with target K562 cells at 1:1 and 2:1 E:T ratios in a 96-well U-bottom plate in 5 % CO₂ at 37 °C for 4 h. Culture of K562 cells in the absence of NK cells was considered as negative control. Cells were incubated with FITC Annexin V in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. Effector cells were identified by co-staining with a PerCP-labeled murine monoclonal antibody to human CD56. All the treatments were performed in triplicates. The mean values of three replicates for each sample were used in the analysis.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The cell culture supernatants of target cell-stimulated UCB-CD34⁺-derived NK and PB NK cells were collected and the level of IFN-γ cytokine was analyzed using enzyme-linked immunosorbent assay, which was performed according to the manufacturer's instructions (Human IFN-γ Mini TMB ELISA Development Kit, PeproTech, NJ, USA).

2.10. Statistical analysis

Data are shown as single values, means as center values and error bars for the standard deviation (SD). The ordinary one-way ANOVA test and nonparametric ANOVA with posthoc Tukey multiple comparison tests was used to statistically compare the marker expression between two treated groups and control. Statistical analyses were performed using GraphPad Prism 7 software. The analyses of flow cytometry data were done by FlowJo (7.6.1) software. The significance level was 0.05 and the corresponding confidence level was 95 %.

3. Results

3.1. Expansion and differentiation of UCB-CD34⁺ cells into NK cells

A protocol based on sequential exposure to different cytokines cocktails was used to facilitate NK cell generation from UCB-CD34⁺ cells. Experiments were performed on UCB-CD34⁺ cells collected from independent donors. The purity of the isolated CD34⁺ cells was analyzed by flow cytometry and it was more than 90 % (data not shown). The HSC structure was round and small, as revealed by microscopic observations (Fig. 1A). We found that proliferation increased substantially in CD34⁺ cells during 2 weeks (Fig. 1B).

Our findings indicated that the CD56⁺ NK cell population appears in the second week of the differentiation phase. The microscopic observations of NK cells obtained through differentiation revealed cells that comprise colonies (Fig. 1C). Fig. 2A shows a schematic representation of the procedure used for the ex vivo generation of NK cells from UCB-CD34⁺ HSCs. Although we were able to successfully generate CD56⁺ NK cells from UCB-CD34⁺ cells, the amount of differentiation varied considerably between donors and there was a delay in the generation of NK cells in donor 3 (Fig. 2B). The percentage of NK cells obtained on day 28 from donor 1, 2 and 3 was 93.9 %, 85.8 %, and 38.4 %, respectively. Neither CD34⁺ cells nor CD3⁺ T cells were detected after the differentiation phase.

3.2. Isolation and expansion of PB CD56⁺CD3⁻ NK cells

According to the expression of CD16 (FcγRIIIa) and the density of CD56 on the cell surface, two NK cell subsets in humans have been identified. About 90 % of circulating NK cells are CD56^{dim} CD16^{bright}, while only 10 % are CD56^{bright} CD16^{-dim} NK cells. Our results were consistent with this (data not shown).

3.3. Flow cytometry analysis of surface expression of UCB-CD34⁺-derived NK and PB NK cells receptors and markers

3.3.1. NKG2D, CD69, and FasL expression

Inhibitory and activating receptors, which can engage ligands on potential target cells, contribute to NK cell-mediated cytotoxicity. Therefore, our next step was to compare the expression of activating and inhibitory receptors in UCB-CD34⁺-derived NK cells and PB NK cells during co-culture with K562 cells at 1:1 and 2:1 E:T ratios by flow cytometry.

The expression of activating receptor NKG2D was significantly increased in UCB-CD34⁺-derived NK cells at 1:1 E:T ratio compared to negative control (53.33 ± 11.02 % vs. 32.10 ± 4.161 %; $p < 0.05$, respectively), 95%CI [11.47,80.7] (Fig. 3A). In PB NK cells, both 1:1 and 2:1 E:T ratios showed significant increase in NKG2D expression compared to negative control (17.07 ± 1.102 % vs. 11.07 ± 2.577 % and 17.83 ± 1.457 % vs. 11.07 ± 2.577 %; $p < 0.05$ and $p < 0.01$, respectively), 95%CI [4.66,21.45] (Fig. 3B). Comparing two NK cell groups, we found that at 1:1 E:T ratio UCB-CD34⁺-derived NK cells express more NKG2D than PB NK cells and the difference was significant (53.33 ± 11.02 % vs. 17.07 ± 1.102 %; $p < 0.001$, respectively), 95%CI [11.47,80.7] (Fig. 3C). Fig. 3D shows a comparison of representative flow cytometry histogram plots of expression of NKG2D at 1:1 and 2:1 E:T ratios in UCB-CD34⁺-derived NK cells and PB NK cells.

As an activation marker of the NK cells CD69 expression in UCB-CD34⁺-derived NK cells increased significantly at both E:T ratios compared to negative control (13.30 ± 1.300 % vs. 2.867 ± 1.422 % and 15.03 ± 3.653 % vs. 2.867 ± 1.422 %; $p < 0.01$, respectively), 95%CI [-0.6,24.1] (Fig. 3E). In PB NK cells only 2:1 E:T ratio showed significant increase in CD69 expression compared to negative control (7.467 ± 1.258 % vs. 3.267 ± 0.7638 %; $p < 0.01$, respectively), 95%CI [1.36,10.59] (Fig. 3F). The expression of CD69 considerably increased in UCB-CD34⁺-derived NK cells as compared to PB NK cells at 1:1 and 2:1 E:T ratios (13.30 ± 1.300 % vs. 5.267 ± 1.124 % and 15.03 ± 3.653 % vs. 7.467 ± 1.258 %; $p < 0.01$ and $p < 0.05$, respectively), 95%CI [2.47,24.1] (Fig. 3G). Fig. 3H shows a comparison of representative flow cytometry histogram plots of expression of CD69 at 1:1 and 2:1 E:T ratios in UCB-CD34⁺-derived NK cells and PB NK cells.

In comparison to the negative control, the expression of FasL considerably increased in UCB-CD34⁺-derived NK cells at both E:T ratios (36.27 ± 9.390 % vs. 5.433 ± 4.163 % and 36.50 ± 8.577 % vs. 5.433 ± 4.163 %; $p < 0.01$, respectively), 95%CI [-4.9,59.5]

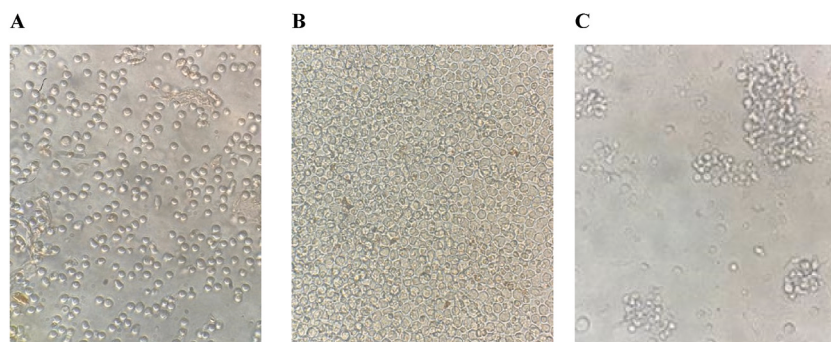


Fig. 1. Morphological structure of CD34⁺ cells and UCB-CD34⁺-derived NK cells. The morphologies of the isolated CD34⁺ cells before and after expansion (A and B), and UCB-CD34⁺-derived NK cells (C).

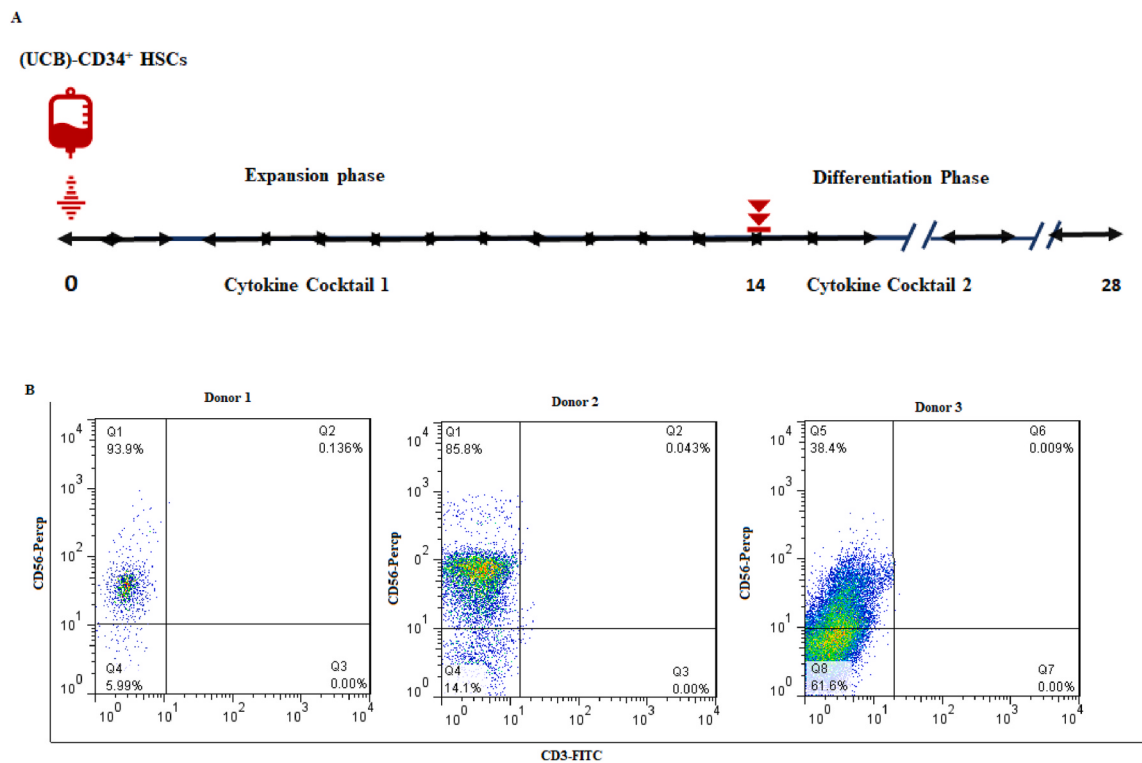


Fig. 2. Ex vivo generation of natural killer (NK) cells from expanded umbilical cord blood (UCB)-CD34⁺ HSCs. (A) Schematic representation of the procedure used for ex vivo generation of NK cells from UCB-CD34⁺ HSCs. The culture protocol includes an initial expansion phase of up to 14 days in serum-free stem cell growth medium containing stem cell growth factor (SCF), recombinant human fms-like tyrosine kinase 3 ligand (FLT3L), recombinant human IL-6, and recombinant human thrombopoietin (TPO) (cytokine cocktail 1). Then we differentiated UCB-CD34⁺ HSCs to NK cells for 2 weeks in NK MACS basal medium with 1 % NK MACS supplement containing SCF, IL-7, FLT3L, IGF-1, IL-15, and IL-2 (cytokine cocktail 2). (B) The percentage of UCB-CD34⁺-derived NK cells on day 28. CD56⁺ NK cells were determined by flow cytometry using FITC-conjugated anti-human CD3 and PerCP-conjugated anti-human CD56 (representative figure of three independent experiments).

(Fig. 3D). FasL expression at both E:T ratios increased in PB NK cells, but this increase was not statistically significant compared to negative control (Fig. 3J). Expression of FasL increased in UCB-CD34⁺-derived NK cells at both 1:1 and 2:1 E:T ratios compared to PB NK cells but this increase was only significant at 1:1 E:T ratio ($36.27 \pm 9.390\%$ vs. $16.63 \pm 3.669\%$; $p < 0.05$, respectively), 95%CI [7.2,59.5] (Fig. 3K). Fig. 3L shows a comparison of representative flow cytometry histogram plots of expression of FasL at 1:1 and 2:1 E:T ratios in UCB-CD34⁺-derived NK cells and PB NK cells.

3.3.2. Natural cytotoxicity receptors (NCRs) expression

Expression of the natural cytotoxicity receptor (NCR), NKp30, in UCB-CD34⁺-derived NK cells decreased at both E:T ratios compared to negative control and this reduction was significant at 1:1 E:T ratio ($17.73 \pm 2.610\%$ vs. $35.43 \pm 5.700\%$; $p < 0.05$, respectively), 95%CI [5.1,49.5] (Fig. 4A). Conversely, NKp30 expression was significantly increased in PB NK cells at both E:T ratios when compared to negative control and 2:1 E:T ratio showed a significant increase in expression of NKp30 compared to 1:1 E:T ratio ($22.00 \pm 2.800\%$ vs. $8.167 \pm 2.290\%$, $35.33 \pm 7.572\%$ vs. $8.167 \pm 2.290\%$ and $35.33 \pm 7.572\%$ vs. $22.00 \pm 2.800\%$; $p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively), 95%CI [2.4,54.1] (Fig. 4B). Comparing UCB-CD34⁺-derived NK cells to PB NK cells, there was no significant difference in NKp30 expression at 1:1 and 2:1 E:T ratios (Fig. 4C). Fig. 4D shows a comparison of representative flow cytometry histogram plots of expression of NKp30 at 1:1 and 2:1 E:T ratios in UCB-CD34⁺-derived NK cells and PB NK cells.

Also, we found that at both E:T ratios UCB-CD34⁺-derived NK cells expressed another NCR, NKp44, but this increase did not reach statistical significance compared to negative control (Fig. 4E). The expression of this receptor in PB NK cells at both E:T ratios was significantly increased compared to negative control ($15.80 \pm 1.513\%$ vs. $4.533 \pm 1.102\%$ and $18.40 \pm 3.305\%$ vs. $4.533 \pm 1.102\%$; $p < 0.01$ and $p < 0.001$, respectively), 95%CI [1.7,26.6] (Fig. 4F). UCB-CD34⁺-derived NK cells showed significantly higher expression of this receptor compared to PB-derived NK cells at both E:T ratios ($46.70 \pm 9.814\%$ vs. $15.80 \pm 1.513\%$ and $46.50 \pm 8.525\%$ vs. $18.40 \pm 3.305\%$; $p < 0.01$, respectively), 95%CI [10.1,71.08] (Fig. 4G). Fig. 4H shows a comparison of representative flow cytometry histogram plots of expression of NKp44 at 1:1 and 2:1 E:T ratios in UCB-CD34⁺-derived NK cells and PB NK cells.

Moreover, at both E:T ratios, NKp46 NCR expression increased significantly in UCB-CD34⁺-derived NK cells compared to negative control ($70.70 \pm 12.70\%$ vs. $41.87 \pm 8.201\%$ and $69.50 \pm 11.39\%$ vs. $41.87 \pm 8.201\%$; $p < 0.05$, respectively), 95%CI [102.3,21.4] (Fig. 4I). Both E:T ratios did not demonstrate any significant differences in NKp46 expression compared to negative control in PB NK

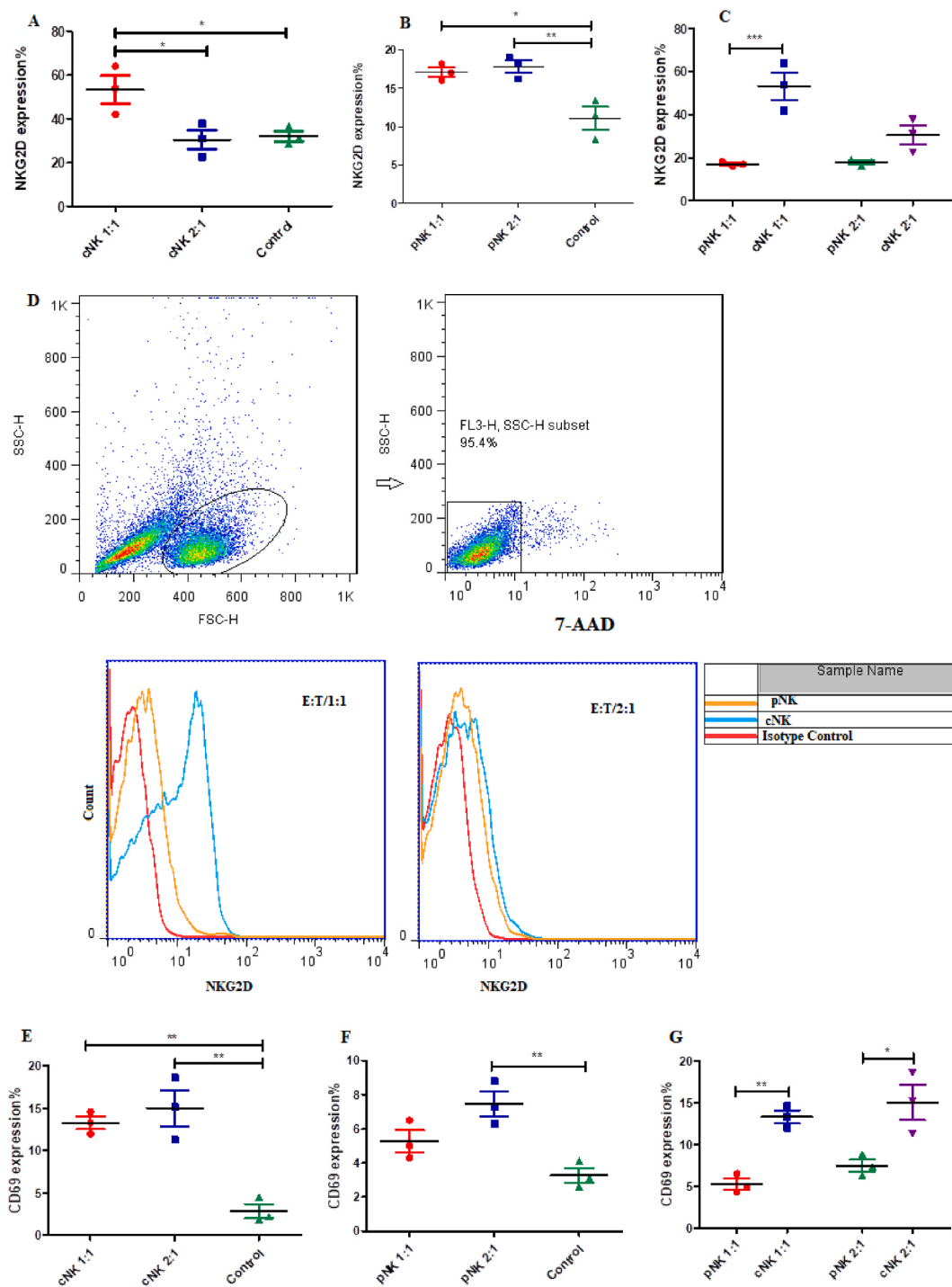


Fig. 3. NK2D, CD69, and FasL expression in UCB-CD34⁺-derived NK cells and PB NK cells after co-culture with K562 cell line and comparison of two NK cell groups. Surface expression of NK2D (A–C), CD69 (E–G), and FasL (I–K) in UCB-CD34⁺-derived NK cells, PB NK cells, and comparison of two NK cell groups at 1:1 and 2:1 E:T ratios, respectively and representative flow cytometry histogram plots (D, H, and L). We considered the culture of UCB-CD34⁺-derived NK cells and PB NK cells alone as a negative control group. Additionally, appropriate isotope controls were used to eliminate non-specific binding. Data are depicted as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001.

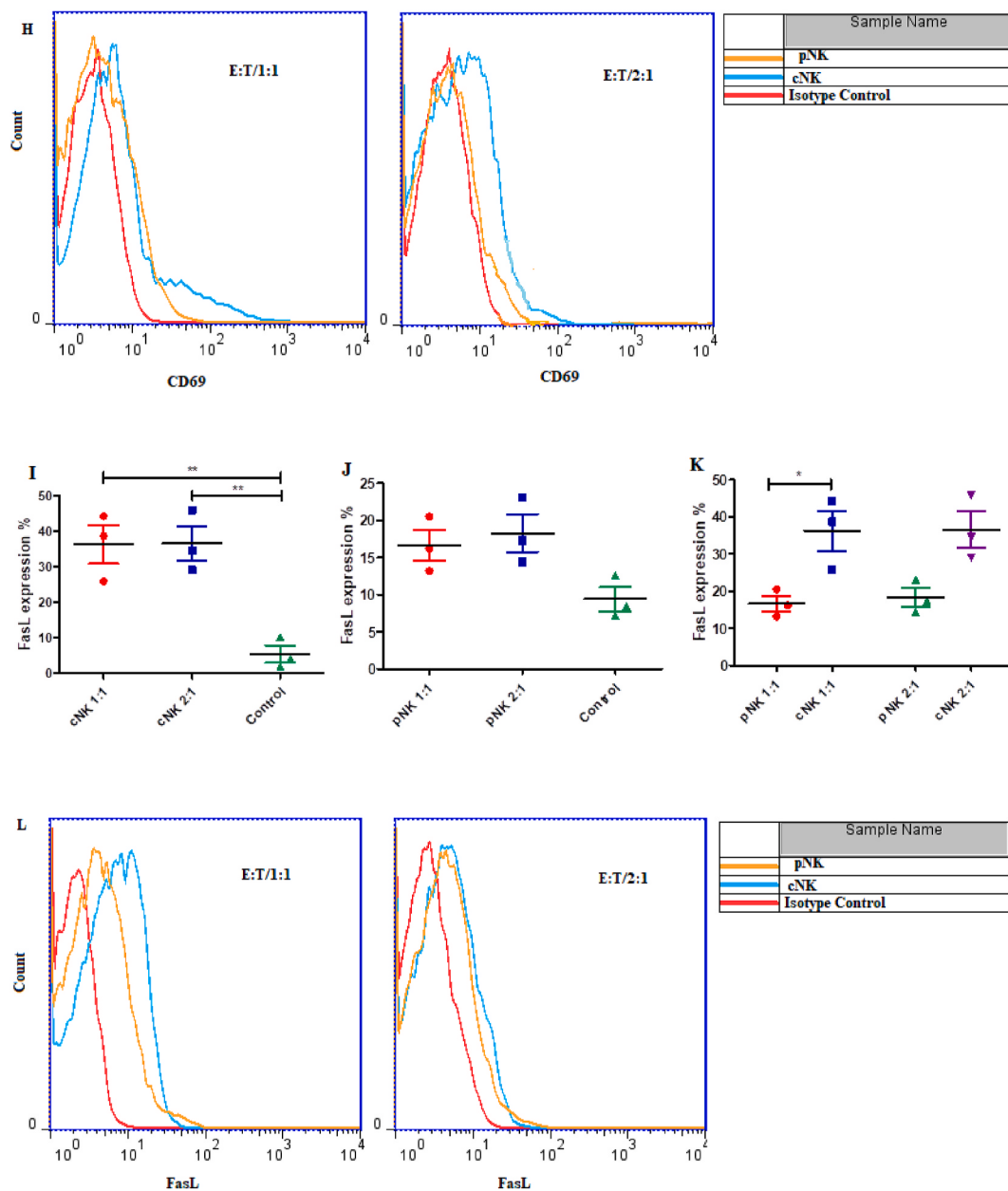


Fig. 3. (continued).

cells (Fig. 4J). The expression of this receptor was found to be significantly higher in UCB-CD34⁺-derived NK cells than in PB NK cells at both E:T ratios (70.70 ± 12.70 % vs. 18.57 ± 0.9074 % and 69.50 ± 11.39 % vs. 19.10 ± 1.082 %; $p < 0.001$, respectively), 95%CI [102.3,16.3] (Fig. 4K). Fig. 4L shows a comparison of representative flow cytometry histogram plots of expression of NKp46 at 1:1 and 2:1 E:T ratio in UCB-CD34⁺-derived NK cells and PB NK cells.

3.3.3. NKG2A, PD1, and CD57 expression

UCB-CD34⁺-derived NK cells at both 1:1 and 2:1 E:T ratios showed significantly lower expression of inhibitory receptor NKG2A compared to negative control (5.333 ± 0.8505 % vs. 16.20 ± 3.100 % and 2.300 ± 1.572 % vs. 16.20 ± 3.100 %; $p < 0.01$ and $p < 0.001$, respectively), 95%CI [-1.6,23.9] (Fig. 5A). However, in PB NK cells there was no significant difference regarding NKG2A expression at both E:T ratios compared to negative control (Fig. 5B). Comparing PB NK cells with UCB-CD34⁺-derived NK cells, we found that at 1:1 E:T ratio, there was no significant difference in NKG2A expression between PB NK cells and UCB-CD34⁺-derived NK cells (Fig. 5C). However, at 2:1 E:T ratio PB NK cells expressed significantly increased NKG2A compared to UCB-CD34⁺-derived NK cells (12.23 ± 3.656 % vs. 2.300 ± 1.572 %; $p < 0.01$, respectively), 95%CI [-1.6,21.3] (Fig. 5C). Fig. 5D shows a comparison of

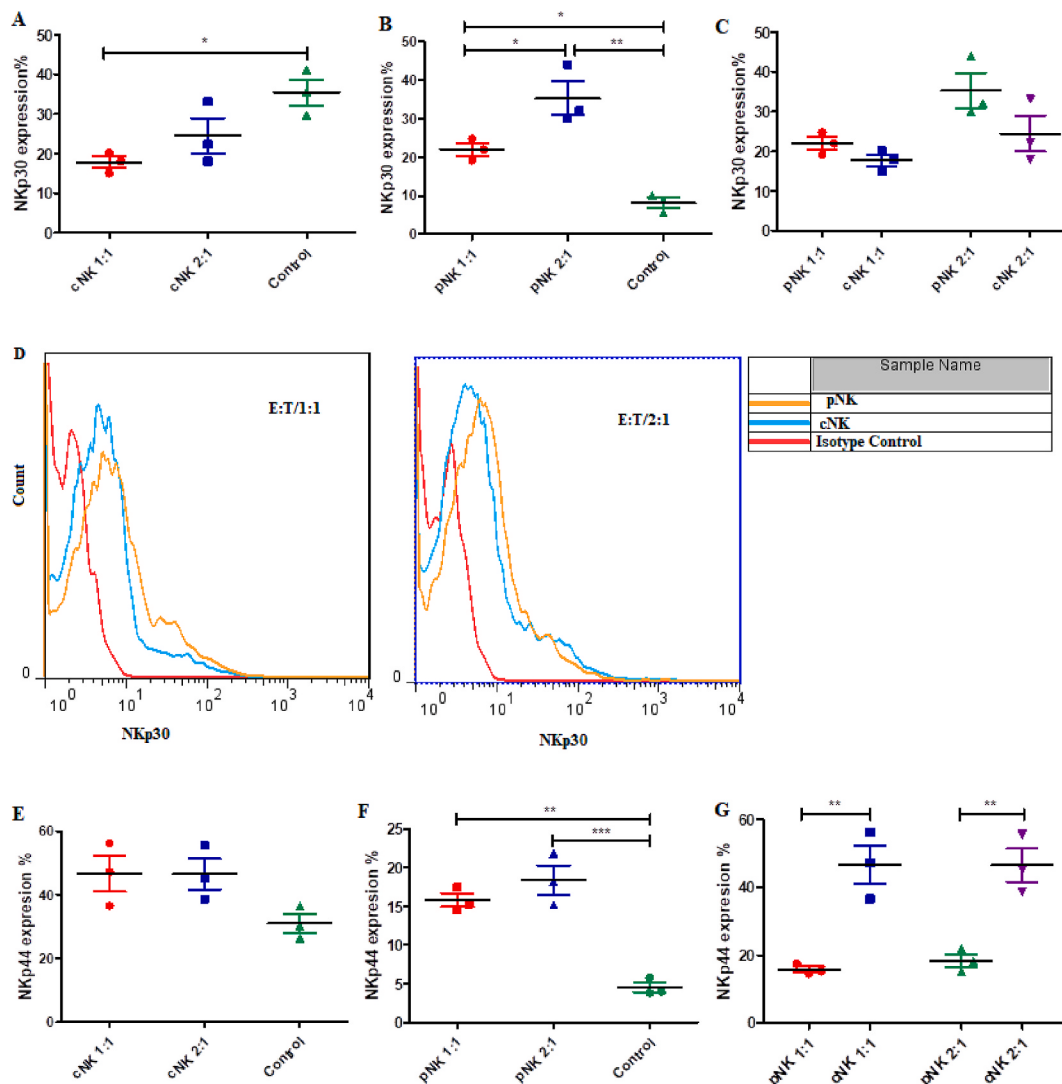


Fig. 4. NKp30, NKp44, and NKp46 expression in UCB-CD34⁺-derived NK cells and PB NK cells after co-culture with K562 cell line and comparison of two NK cell groups. Surface expression of NKp30 (A–C), NKp44 (E–G), and NKp46 (I–K) in UCB-CD34⁺-derived NK cells, PB NK cells, and comparison of two NK cell groups at 1:1 and 2:1 E:T ratios, respectively and representative flow cytometry histogram plots (D, H, and L). We considered the culture of UCB-CD34⁺-derived NK cells and PB NK cells alone as a negative control group. Additionally, appropriate isotype controls were used to eliminate non-specific binding. Data are depicted as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

representative flow cytometry histogram plots of expression of NKG2A at 1:1 and 2:1 E:T ratios in UCB-CD34⁺-derived NK cells and PB NK cells.

We also assessed the expression of the inhibitory receptor, PD-1, and we found that PD1 expression in UCB-CD34⁺-derived NK cells was not changed significantly at both E:T ratios compared to negative control (Fig. 5E). The expression of this receptor in PB NK cells was lower at both E:T ratios compared to negative control, and this decrease was significant at 1:1 E:T ratio compared to the negative control ($10.80 \pm 3.100\%$ vs. $26.83 \pm 6.732\%$; $p < 0.05$, respectively), 95%CI [3.09,43.5] (Fig. 5F). When UCB-CD34⁺-derived NK cells compared to PB NK cells at 1:1 and 2:1 E:T ratios, there was no significant change regarding PD-1 expression (Fig. 5G). Fig. 5H shows a comparison of representative flow cytometry histogram plots of expression of PD-1 at 1:1 and 2:1 E:T ratios in UCB-CD34⁺-derived NK cells and PB NK cells.

When compared to the negative control, the expression of CD57, which is a marker of NK cell maturation, in UCB-CD34⁺-derived NK cells and PB NK cells did not differ significantly at either ratio compared to their controls (Fig. 5I and J), but its expression in PB NK cells was increased significantly when compared to UCB-CD34⁺-derived NK cells at both E:T ratios ($53.14 \pm 7.181\%$ vs. $12.80 \pm 4.503\%$ and $42.17 \pm 10.55\%$ vs. $8.433 \pm 4.114\%$; $p < 0.001$ and $p < 0.01$, respectively), 95%CI [−1.7,70.9] (Fig. 5K). Fig. 5L shows a comparison of representative flow cytometry histogram plots of expression of CD57 at 1:1 and 2:1 E:T ratios in UCB-CD34⁺-derived NK

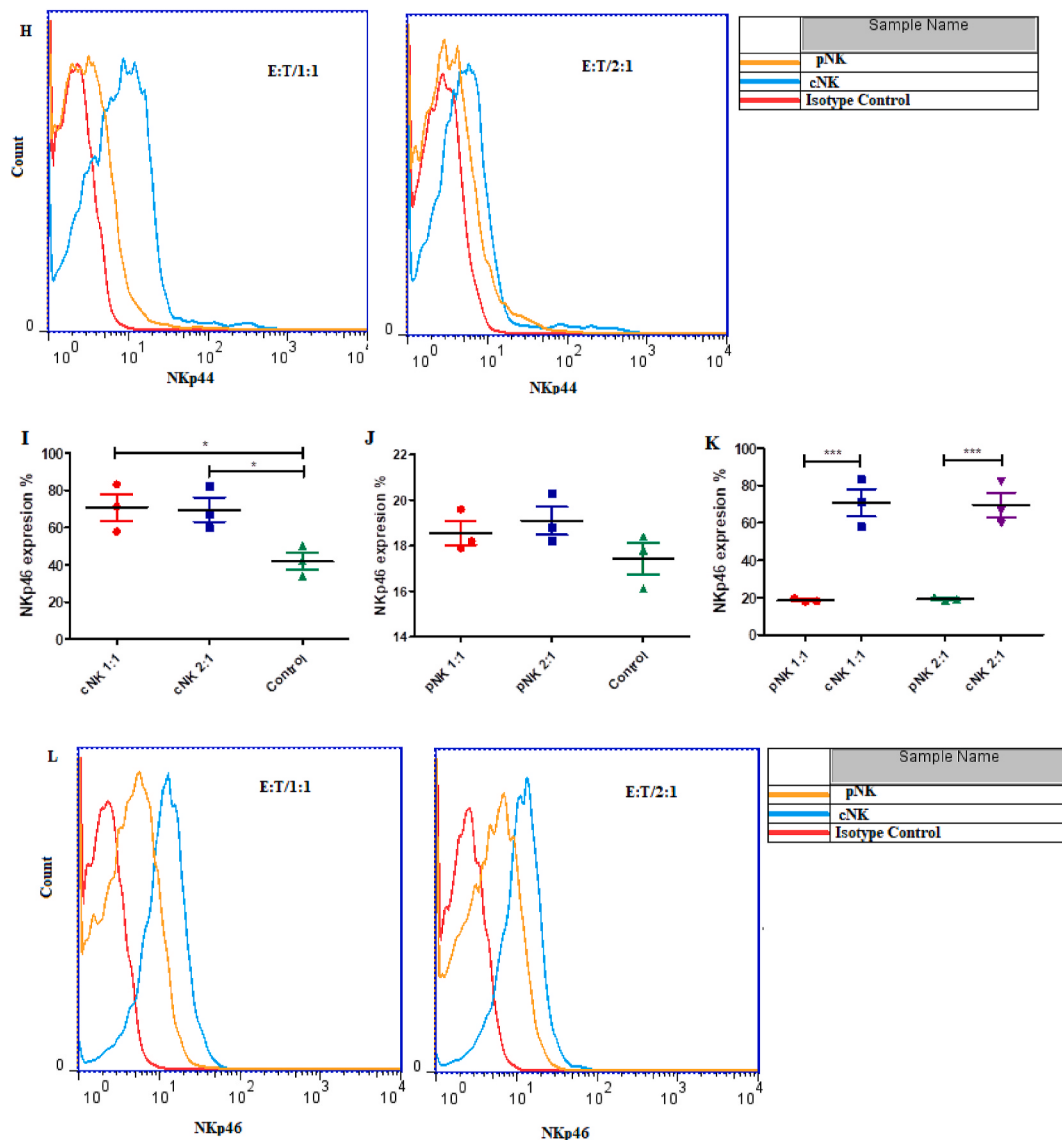


Fig. 4. (continued).

cells and PB NK cells.

3.4. CD107a degranulation of UCB-CD34⁺-derived NK and PB NK cells

CD107a-based degranulation assays using K562 as target cells showed that at 1:1 and 2:1 E:T ratios, the significant increase in cytolytic activity was observed in UCB-CD34⁺-derived NK cells compared to negative control however this increase was more at 1:1 E:T ratio compared to 2:1 E:T ratio ($35.77 \pm 10.82\%$ vs. $2.233 \pm 1.206\%$ and $23.63 \pm 5.415\%$ vs. $2.233 \pm 1.206\%$; $p < 0.01$ and $p < 0.05$, respectively), 95%CI $[-0.76, 62.6]$ (Fig. 6A). PB NK cells did not show any significant difference at 1:1 E:T ratio compared to negative control but 2:1 E:T ratio showed significantly increased expression ($12.03 \pm 3.787\%$ vs. $3.200 \pm 1.212\%$; $p < 0.05$, respectively), 95%CI $[0.18, 23.6]$ (Fig. 6B). When comparing UCB-CD34⁺-derived NK cells to PB NK cells at 1:1 and 2:1 E:T ratios, it was shown that UCB-CD34⁺-derived NK cells at 1:1 E:T ratio significantly expressed CD107a more than PB NK cells ($35.77 \pm 10.82\%$ vs. $6.733 \pm 1.650\%$; $p < 0.01$, respectively), 95%CI $[2.6, 62.6]$ (Fig. 6C). Fig. 6D and E shows percentage of CD56⁺CD107a⁺ UCB-CD34⁺-derived NK cells and PB NK cells at 1:1 and 2:1 E:T ratios, respectively.

3.5. Apoptosis induction by UCB-CD34⁺-derived NK and PB NK cells

To further assess the NK-mediated cytotoxicity, FITC-Annexin V staining was performed to measure the induction of apoptosis in

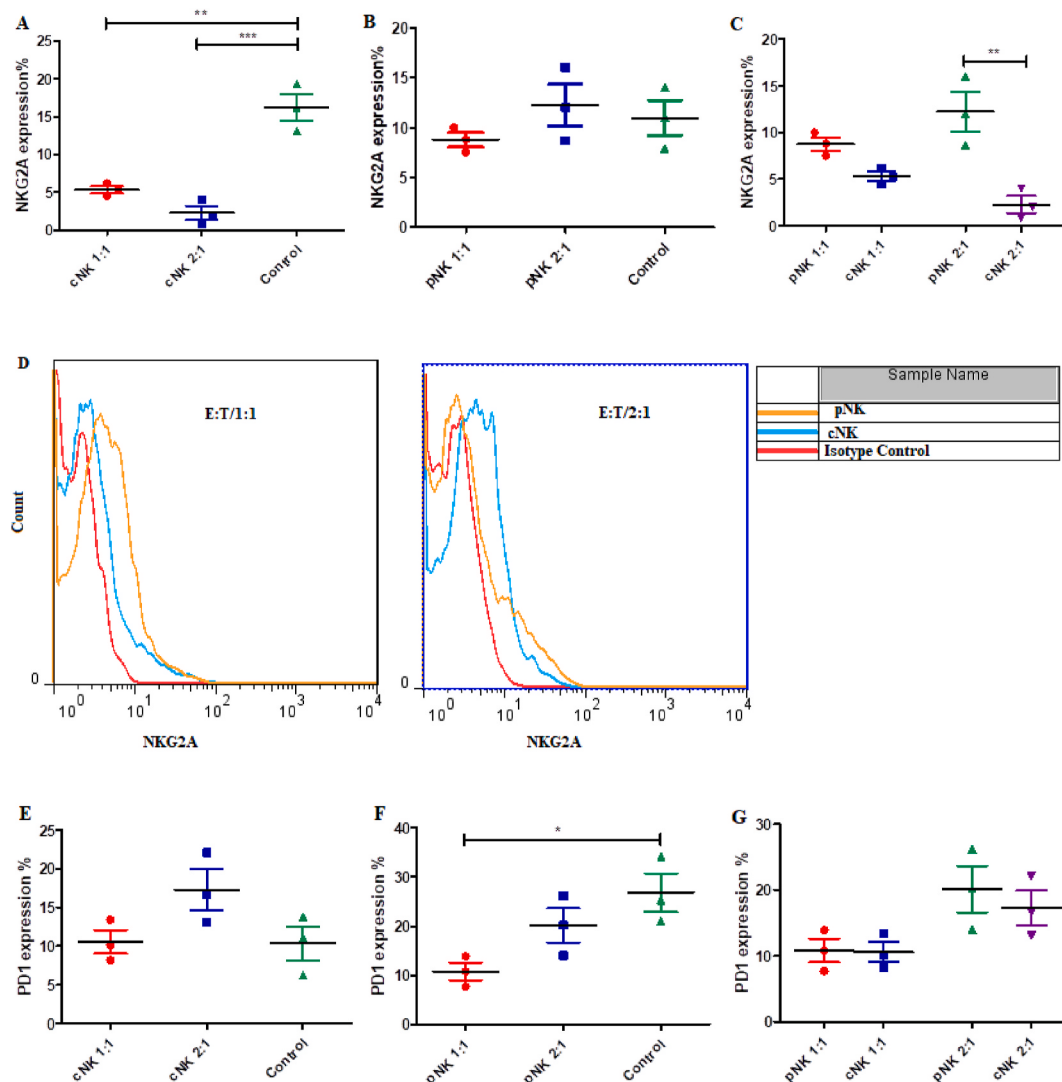


Fig. 5. NKG2A, PD-1, and CD57 expression in UCB-CD34⁺-derived NK cells and PB NK cells after co-culture with K562 cell line and comparison of two NK cell groups. Surface expression of NKG2A (A–C), PD-1 (E–G), and CD57 (I–K) in UCB-CD34⁺-derived NK cells, PB NK cells, and comparison of two NK cell groups at 1:1 and 2:1 E:T ratios, respectively and representative flow cytometry histogram plots (D, H, and L). We considered the culture of UCB-CD34⁺-derived NK cells and PB NK cells alone as a negative control group. Additionally, appropriate isotope controls were used to eliminate non-specific binding. Data are depicted as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

the target cells. At 1:1 and 2:1 E:T ratios, a significant increase in apoptosis was observed in UCB-CD34⁺-derived NK cells compared to the negative control (65.90 ± 6.003 % vs. 10.60 ± 0.8888 % and 54.97 ± 9.058 % vs. 10.60 ± 0.8888 %; $p < 0.001$, respectively), 95% CI [8.3,80.8] (Fig. 7A). Similar results were observed for PB NK cells (63.87 ± 8.834 % vs. 16.70 ± 5.742 % and 67.63 ± 8.792 % vs. 16.70 ± 5.742 %; $p < 0.001$, respectively), 95%CI [2.4,89.4] (Fig. 7B). There was no noticeable difference between UCB-CD34⁺-derived NK cells and PB NK cells when comparing the 1:1 and 2:1 E:T ratios (Fig. 7C).

3.6. IFN- γ production by UCB-CD34⁺-derived NK and PB NK cells

The amount of IFN- γ that the UCB-CD34⁺-derived NK cells and PB NK cells secreted, in response to K562 cells, was assessed. The IFN- γ level did not differ significantly in UCB-CD34⁺-derived NK cells and PB NK cells at both E:T ratios compared to their controls (Fig. 8A and B). When comparing UCB-CD34⁺-derived NK to PB NK cells at 1:1 and 2:1 E:T ratios, there was no significant difference in IFN- γ production (Fig. 8C).

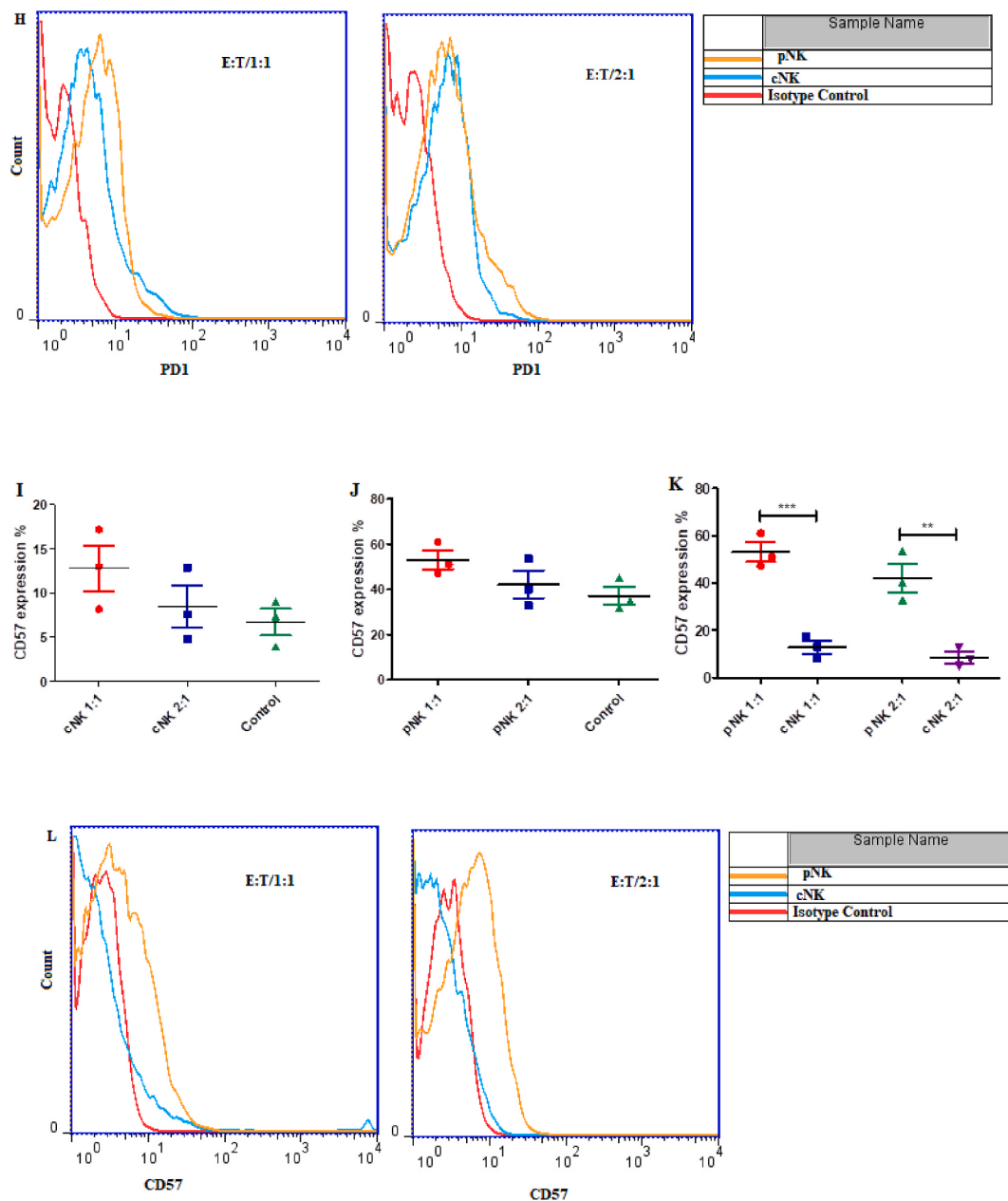


Fig. 5. (continued).

4. Discussion

UCB, a source of $CD34^+$ HSCs, has attracted attention as an allogenic and off-the-shelf product for NK cell differentiation [18,19]. Expansion of functional NK cells from UCB- $CD34^+$ cells using various methods has been accomplished [20,21]. Previous preclinical and clinical studies have shown promising results for UCB- $CD34^+$ -derived NK cell-based therapies [22–24]. In the present study, we expanded NK cells from UCB- $CD34^+$ stem cells by a two-step protocol, consisting of two weeks of $CD34^+$ stem cell proliferation and two weeks of NK cell differentiation treated with SCF, FLT3L, IL-7, IGF-1, IL-2, and IL-15. Also, we isolated PB NK cells and cultured them for one week in the presence of IL-2 and IL-15. We compared the phenotypes of UCB- $CD34^+$ -derived NK cells with PB NK cells in the presence of K562 target cells.

We found that the percentage of NK cells after differentiation was different between UCB samples. Consistent with our findings, previous studies have indicated some donors are considered poor responders, and in this state treatment of cells with feeder cells improves NK cell development [17].

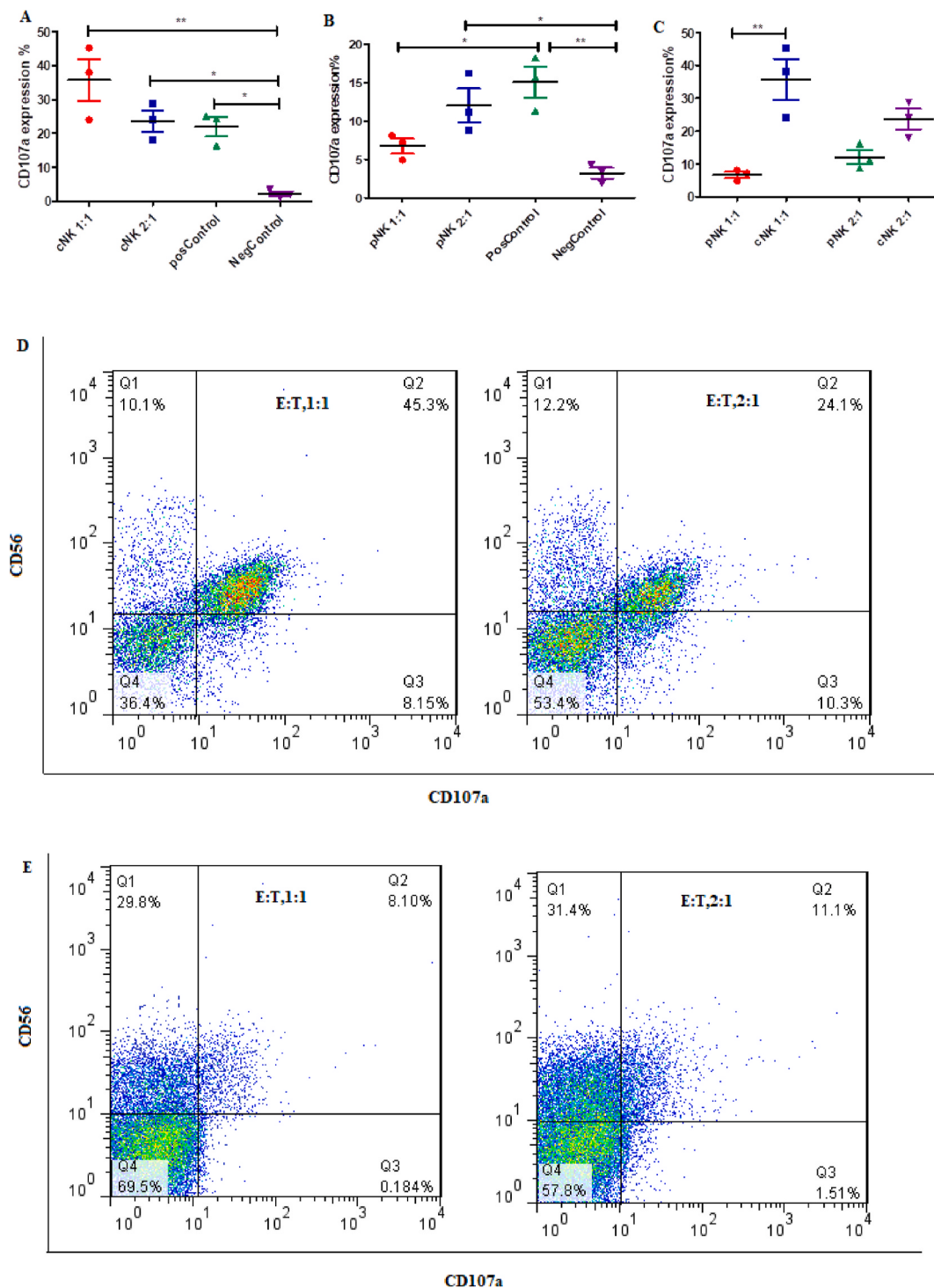


Fig. 6. CD107a degranulation in UCB-CD34⁺-derived NK cells and PB NK cells after co-culture with K562 cell line and comparison of two NK cell groups. CD107a degranulation in UCB-CD34⁺-derived NK cells, PB NK cells, and comparison of two NK cell groups at 1:1 and 2:1 E:T ratios, respectively (A–C) and representative flow cytometry plots (D and E). UCB-CD34⁺-derived NK cells and PB NK cells were left untreated as negative control. Data are depicted as mean $\pm$ SD. *p < 0.05, **p < 0.01.

According to our findings, this established ex vivo differentiation method is overall robust and valid and has the potential to generate NK cells coherently. After two weeks of differentiation, resulting NK cells exhibited a mature and functional phenotype, referred to as CD56^{dim} expression in high with absent or relatively low levels of CD16 and expression of NK-cell-associated cell surface

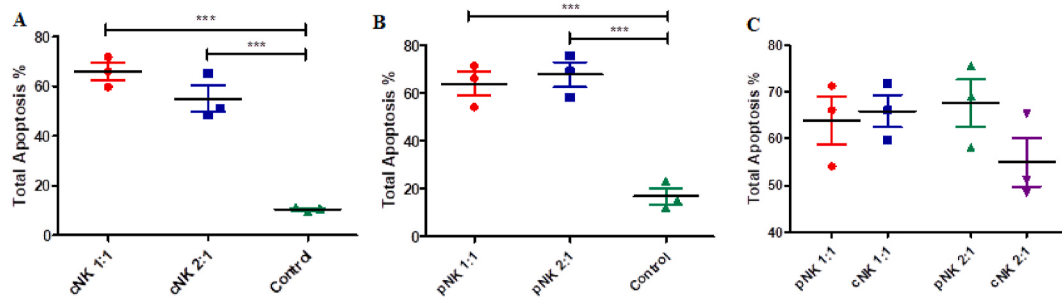


Fig. 7. Apoptosis induction by UCB-CD34⁺-derived NK cells and PB NK cells after co-culture with K562 cell line and comparison of two NK cell groups. Apoptosis induction by UCB-CD34⁺-derived NK cells, PB NK cells, and comparison of two NK cell groups at 1:1 and 2:1 E:T ratios, respectively (A–C). K562 cells were left untreated as negative control. Data are depicted as mean $\pm$ SD. ***p < 0.001.

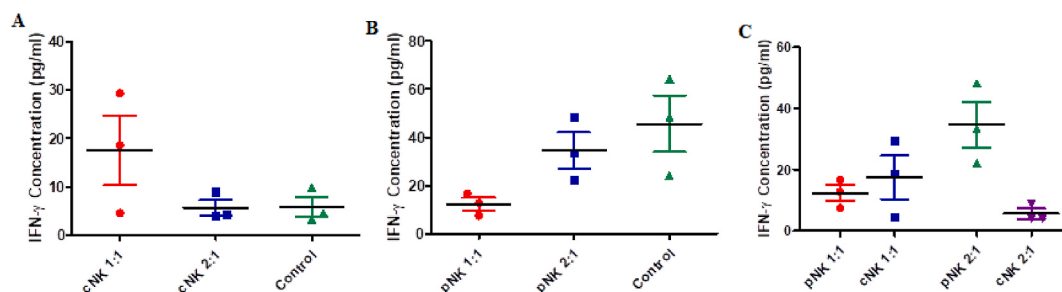


Fig. 8. IFN- γ production by UCB-CD34⁺-derived NK cells and PB NK cells after co-culture with K562 cell line and comparison of two NK cell groups. IFN- γ production by UCB-CD34⁺-derived NK cells, PB NK cells, and comparison of two NK cell groups (A–C). We considered the culture of UCB-CD34⁺-derived NK cells and PB NK cells alone as a negative control group. Data are depicted as mean $\pm$ SD.

markers, including NKG2A, NKG2D, NKP30, NKP44, and NKP46. Also, we compared the expression of NK cell receptors between UCB-CD34⁺-derived NK cells and PB NK cells after co-culture with the K562 cell line as target cells in two E:T ratios.

Consistent with our findings lower expression of CD16 has been indicated in UCB-CD34⁺-derived and iPSC-derived NK cells as compared to UCB-derived mature NK cells [25]. Also, in the present study, we expanded UCB-CD34⁺-derived NK cells in the presence of IL-2 and IL-15. Several studies have reported treatment of UCB-CD34⁺-derived NK cells with IL-2 and IL-15 improves the cytotoxicity against leukemia cells [26,27].

According to our findings, UCB-CD34⁺-derived NK cells at both E:T ratios (1:1 and 2:1) expressed lower levels of inhibitory receptor NKG2A compared to negative control. Also, at a 2:1 E:T ratio, PB NK cells expressed significantly higher NKG2A than UCB-CD34⁺-derived NK cells. NKG2A as one of the inhibitory receptors plays an important role in regulating NK cell's responses against target cells and limits the cytotoxic function of NK cells. Luevano et al. have indicated higher expression of NKG2A in cord blood NK cells and lower levels of CD16, KIRs, and DNAM-1 compared to PB NK cells related to their immaturity [28]. Therefore, lower expression of NKG2A in UCB-CD34⁺-derived NK cells in the present study confirms the maturity and activity of these cells.

Moreover, we found that at a 1:1 E:T ratio, UCB-CD34⁺-derived NK cells significantly express more NKG2D than PB NK cells. Our results support previous findings where upregulation of NKG2D upon NK cell's engagement with target cells mediate NK cell activation, a statement that has been shown in UCB-CD34⁺-derived NK cells [29–31].

Expression of the NCR, NKP30, in UCB-CD34⁺-derived NK cells decreased at both E:T ratios compared to the negative control. Conversely, NKP30 expression was significantly increased in PB NK cells at both E:T ratios. However, there was no significant difference in NKP30 expression between UCB-CD34⁺-derived NK cells and PB NK cells. Also, we found that UCB-CD34⁺-derived NK cells showed significantly higher expression of NKP44 and NKP46 receptors compared to PB-derived NK cells at both E:T ratios. NKP30, NKP44, and NKP46 belong to the NCR family of activating receptors and recognize different ligands of target cells [32]. In Oberoi et al. [17] study NKP30 expression in ex-vivo-generated NK cells has indicated a heterogenic pattern between donors. Therefore, no difference in NKP30 expression between UCB-CD34⁺-derived NK cells and PB NK cells may be due to this issue. Also, they have shown that NK cells exposed to K562/41BBL/mb15/mb21 cells express significantly lower levels of NKP30 and NKP44 than NK cells differentiated from PB-CD34⁺ cells and only expanded in the presence of cytokines [17]. Altogether higher expression of activating receptors in UCB-CD34⁺-derived NK cells compared to PB NK cells indicates the strength of activation in these cells.

In the assessment of CD57 as a differentiation marker in NK cells, we found that PB NK cells expressed significantly higher CD57 compared to UCB-CD34⁺-derived NK cells. Studies have documented that expression of this molecule elevates with age [33,34]. Brenchley et al. [35] in a study have shown CD57 expression is related to replicative senescence and proposed CD57 as a marker for the definition of highly differentiated NK cells. Furthermore, Bjorkstrom et al. [36] have suggested CD57 expression is associated with

weak proliferative capacity and has a negative effect on IFN- γ production where our results in PB NK cells support these findings.

NK cell's cytotoxic function is mediated through two main pathways, including perforin/granzyme B and the FasL [37]. In the present study, expression of FasL increased dramatically in UCB-CD34⁺-derived NK cells at both E:T ratios compared to PB NK cells. FasL expression has not been detected in the cord blood-CD34⁺-derived NK in the Carayol et al. [38] study. IL-2 stimulation induces FasL expression in NK cells; therefore, IL-2 treatment during UCB-CD34⁺-derived NK expansion justifies the FasL expression in our study.

As a point of departure, we analyzed the expression of CD69, CD107a, and IFN- γ as the indicators of NK cell activation. CD69 expression was evaluated and our findings indicated that CD69 expression in UCB-CD34⁺-derived NK cells increased significantly at both E:T ratios compared to negative control. Also, the expression of CD69 considerably increased in UCB-CD34⁺-derived NK cells as compared to PB NK cells at both E:T ratios. Also, in comparing UCB-CD34⁺-derived NK cells to PB NK cells at 1:1 and 2:1 E:T ratios, it was shown that UCB-CD34⁺-derived NK cells at 1:1 E:T ratio significantly expressed CD107a more than PB NK cells. Moreover, while IFN- γ level was increased at 1:1 E:T ratio in UCB-CD34⁺-derived NK cells compared to control, it was not statistically significant. The level of IFN- γ in PB NK cells was decreased compared to control, but it was not significant. Also, when comparing UCB-CD34⁺-derived NK to PB NK cells at 1:1 and 2:1 E:T ratios, there was no significant difference in IFN- γ production. According to Bjorkstrom et al. [36] study, due to their poor proliferative capacity, CD57⁺CD56^{dim} NK cells stimulated with IL-12 and IL-15 produce lower levels of IFN- γ . Therefore, the decreased level of IFN- γ in PB NK cells probably is related to the higher levels of CD57 expression.

Oberoi et al. [17] have differentiated NK cells from PB HSCs (PB-CD34⁺ cells) and indicated the cells from different donors, which expanded in the presence of only cytokines show heterogeneous behavior against K562 target cells. However, when they exposed cells with K562/41BBL/mb15/mb21 feeder cells cytotoxicity against K562 target cells increased. Also, Yu et al. [39] have reported that UCB-CD34⁺-derived NK cells alone indicate a weak cytotoxic function against the K562 target cell, while they expanded UCB-CD34⁺-derived NK cells in the presence of hrIL-15 for 4–5 weeks. The discrepancy between our results and these studies may be due to the difference in the source of stem cells and culture duration, respectively. Overall, UCB-CD34⁺ stem cells and UCB-CD34⁺-derived NK cells may serve as a basis for NK therapy and genetic manipulation designed to enhance the antitumor function of NK cells. Accordingly, Ohira et al. recently have evaluated therapeutic impacts of adoptive transfer of NK cells derived from PB CD34⁺ stem cells. They have shown that such treatment is completely safe and has anti-tumor effects in hepatocellular carcinoma recurrence and have decided to continue the study in the next clinical trial phases [40]. In another study Jonge et al. have produced CD34⁺ progenitor-derived NK cells for adoptive immune cell therapy in acute myeloid leukemia (AML) [41].

One of the limitations of our study was that we did not evaluate all NK cell receptors. Also investigating the signaling pathways and other molecules and cytokines related to the activation of NK cells would have contributed to the strength of our work. The present study was an in vitro study and it is necessary to investigate the therapeutic effects of these two types of NK cells in animal models of cancer.

5. Conclusions

Taken together, our data demonstrate that UCB-CD34⁺-derived NK cells expanded and differentiated according to our protocol have a strong cytotoxic function and are a highly functional source of allogeneic NK cells compared to PB for NK cell-based immunotherapy. Furthermore, UCB-CD34⁺ stem cells and UCB-CD34⁺-derived NK cells may serve as a basis for genetic manipulation designed to enhance the antitumor function of NK cells.

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Ethical approval

The study approved by ethical committee of Isfahan University of Medical Sciences (IR.MUI.REC.1400.015).

Data availability

Data will be made available on request.

CRedit authorship contribution statement

Farhoodeh Ghaedrahmati: Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation, Conceptualization. **Nafiseh Esmaeil:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Vajihe Akbari:** Validation, Conceptualization. **Farzaneh Ashrafi:** Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

Abbreviations

NK	natural killer
PB	peripheral blood
HSCs	hematopoietic stem and progenitor cells
UCB	umbilical cord blood
MHC	major histocompatibility complex
hESCs	human embryonic stem cells
iPSCs	induced pluripotent stem cells
PBMCs	peripheral blood mononuclear cells
NCR	natural cytotoxicity receptor
SCF	stem cell growth factor
FLT3L	fms-like tyrosine kinase 3 ligand;
TPO	thrombopoietin
IGF-1	insulin-like growth factor 1
7-AAD	7-Aminoactinomycin D

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