CHAPTER III

EX VIVO EXPANSION OF CORD BLOOD-DERIVED NK CELLS USING FEEDER-FREE AND CELL SORTING-FREE TECHNIQUES

3.1 Abstract

NK cells are members of the innate immune system that play an essential role in eliminating virally-infected, stressed, or cancerous cells in the absence of prior sensitization or antigen presentation. Before initiating cytotoxicity, NK cells employ several types of receptors, through different mechanisms, to identify harmful cells. Moreover, recent evidence reveals that NK cells receive reinforcements from adaptive immune responses to mediate additional cell death. With the aforementioned potentials, adoptive cell transfer (ACT) of *ex vivo* expanded NK cells has now become a promising treatment for virus-borne diseases and cancer. To fulfill these never-ending demands, this study develops a newer strategy to expand NK cells from CB, owing to its advantage of endless supply. To promote CBNK cell proliferation, a newly identified Notch signaling activator Yhhu-3792 or a potent immunopotentiator LPS were supplemented into the culture system during the first week of expansion. The results from flow cytometry analysis show that CB-MNCs can be effectly expanded and give rise to purified CBNK cells under 1 μg/mL LPS stimulation. Upscaling of the expansion reveals that a clinically relevant cell dose of purified functional CBNK cells could be obtained after 21 days of expansion.

3.2 Introduction

ACT of ex vivo expanded cytotoxic lymphocytes has emerged as a promising approach to treat various types of advanced cancer including haematological and solid malignancies. Unlike T cells which require specific antigen recognition and activation, Natural killer (NK) cells are the body's frontline defense that recognizes and efficiently eliminates transformed cells without the need for prior sensitization or human leukocyte antigen (HLA; also known as major histocompatibility complex

(MHC)) matching. Endowed with a wide array of germline-encoded activating and inhibitory receptors, NK cells recognize healthy cells from pathogens and diseased cells, referred to as discrimination of self, non-self, or modified self, respectively (Zucchini et al., 2008). Indeed, NK cells preferentially eliminate virally infected or transformed cells due to the fact that these cells frequently downregulate MHC class I molecules, which are the most important inhibitory ligands for NK cells (for comprehensive overview on NK cell activation mechanisms see Shimasaki *et al.*, 2020). Moreover, in the allogeneic setting, clinical evidence indicates that transfusion of NK cells rarely causes graft versus host disease (GvHD) when compared to T cells (Olson *et al.*, 2010; Shaffer *et al.*, 2016). Therefore, these scenarios make adoptive transfer of allogeneic NK cells an attractive ACT strategy for cancer treatment, named immunotherapy.

Compared to all existing sources of NK cells, cord blood-derived NK (CBNK) cells offer several advantages, including the vast availability of umbilical cord blood (CB) in Cord blood banks worldwide, the absence of safety concerns related to genetically engineered procedures, their non-cancer cell origin, and their high proliferation potential (Heipertz *et al.*, 2021). To avoid the multitude of regulatory concerns arising from the use of feeder cells and the costly cell sorting process, the challenge of using CBNK cells in a clinical setting of cancer immunotherapy lies in developing a feeder-free and cell-sorting-free protocol to generate clinically relevant NK cell doses from CB. To achieve this goal, promoting NK cell development in the early stages of expansion is crucial. Previous studies have shown that activation of Notch signaling regulate NK cell differentiation of $CD34^+$ hematopoietic stem cells (HSCs) (Benne *et al.*, 2009; Haraguchi *et al.*, 2009). Apart from Notch signaling activator, it is demonstrated that bacterial lipopolysaccharides provide beneficial effects on the proliferation (Goodier *et al.*, 2000) and functional activation (Kanevskiy *et al.*, 2019) of peripheral blood derived NK cells (PBNK cells). Therefore, the purpose of this study was to develop an effective feeder-free and cell-sorting-free method for the expansion of purified CBNK cells, aiming to harness their potential for use in cancer immunotherapy.

3.3 Materials and methods

3.3.1 Preparation CB-MNCs

In this study, ethical approval was granted from the Ethics Committee for Researches Involving Human Subjects, Suranaree University of Technology (EC-66-0065), Nakhon Ratchasima, Thailand. With signed informed consents from the parents, CB units (N=15) have been obtained and processed within 12 hours after collection. In all experiments, cord blood derived mononuclear cells (CB-MNCs) were isolated and cultured in the following condition. CB-MNCs were separated using Lymphosep® (Biowest) density gradient centrifugation. First, CB samples were carefully layered on the surface of separation medium, in the ratio of 2:1 (blood:Lymphosep®), in a 50 mL SepMate™ tube (StemCell Technologies). Then, the mixture was centrifuged at 800 × g for 30 minutes at room temperature. Buffy coats containing lymphocytes were collected and washed twice by phosphate buffered saline (PBS) containing 10% acid citric dextrose solution A at 800 × g for 9 minutes and 400 × g for 9 minutes, respectively. Number and viability of the cells were determined by trypan blue dye exclusion staining using an improved Neubauer counting chamber. In all following experiments, the initial seeding density of CB-MNCs was adjusted to 2.0×10^6 cells/ml by dilution in Cellex NKGM-1 medium. Cells were maintained in a humidified atmosphere of 37°C and 5% CO_2 incubator. Fresh medium was added every 3 or 4 days.

3.3.2 LPS and Yhhu-3792 stimulation

For LPS and Yhhu-3792 treatment experiment, CB-MNCs (n = 5) were equally divided into 3 groups and cultured in T75 flask. In all groups, cells were cultured in the basal medium comprising Cellex NKGM-1 medium (KOHJIN BIO) supplemented with 10% human serum, 1,000 IU/ml interleukin 2 (IL-2, Corefront), and 5 μM zoledronic acid (Sigma-Aldrich). LPS (serotype 026:B6; Sigma-Aldrich) and Yhhu-3792 (Tocris Bioscience) were manually added into the cultures to the concentration of 1 μg/ml and 2.5 μM, respectively. Of note, cell cultured in the basal medium were used as control. These cells hereafter were collectively referred to as LPS-, Yhhu-3792- and control group. After 7 days of treatment, LPS and Yhhu-3792 were withdrawn. Then, all groups of cells were cultured in basal medium for another 7 days (Fig. 3.1). The percentage of NK cells were determined on day 0,

day 7 and day 14 by flow cytometry analysis. The condition that yields the highest percentage of NK cells were selected for the concentration optimization experiment.

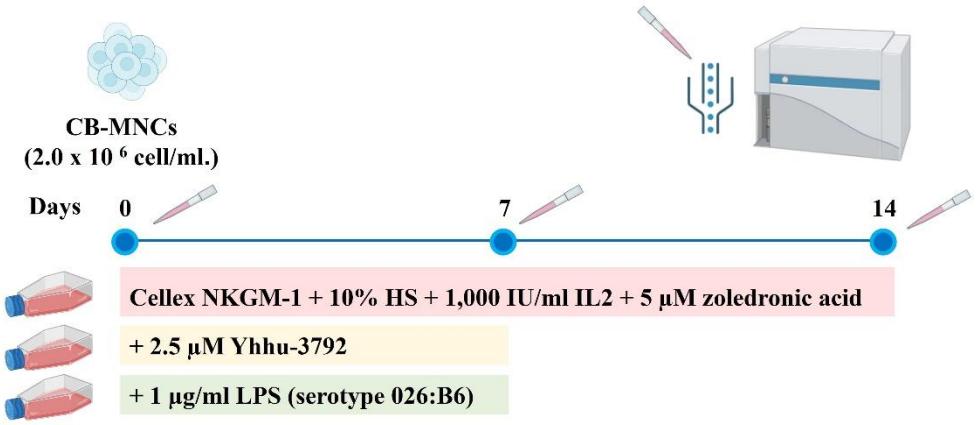

Figure 3.1 The schematic diagram of the experiment used to stimulate the expansion of CBNK cells.

3.3.3 CCK-8 assay

To establish a safety range for the concentration optimization experiment, 2.5 x 10^4 CB-MNCs (n = 5) were seeded in a well of 96-well plates at a volume of 100 µl per well. The cells were then cultured in varying concentrations of the selected supplement. After 48 hours of culture, CCK-8 (Sigma-Aldrich) was added to the well at a ratio of 10 µl CCK-8 per 100 µl sample. The culture was incubated for an additional 4 hours at 37°C under 5% CO_2 before being measured by Azure Biosystems (Dublin, CA, USA) microplate reader. The absorbance intensities were determined at 450 nm. Culture with no supplement was served as the control, while culture with no supplement plus 5% DMSO was served as the death control. Incubated medium was served as blank control. Data were presented as $OD_{test} - OD_{blank}$

3.3.4 Concentration optimization

To obtain an optimal concentration of the selected supplement suited for CBNK cell expansion, various concentrations of the selected supplement (devoid of toxic concentration) were tested using the same procedure as the stimulation experiment (n = 5). Cell cultured in basal medium were used as control. The

percentage of NK cells were determined on day 0, day 7 and day 14. The concentration that yields the highest percentage of NK cells were selected for the upscaling expansion experiment.

3.3.5 Upscaling expansion of CBNK cells

In an attempt to achieve the highest CBNK cell yield, the best conditions from previous experiments were applied (figure 3.2). A minimum of $80x10^6$ CB-MNCs, with a maximum volume of 50 ml per bag, was required as the initial starting population, n = 5. During the first 7 days of culture, cells were cultured in an initiation culture bag (Cellex, Japan). After that, cells were transferred to two CO_2-permeable expansion culture bags (1 L medium/bag, Cellex, Japan). During day 7 to day 21, approximately 150-200 ml of fresh NKGM-1 medium plus 150,000 IU IL-2 were added into an individual bag at 3- to 4-day intervals. On day 0 and day 21, the number of total nucleated cells (TNCs) was determined by trypan blue staining. Fold expansion of the cells was calculated by dividing the number of the cells on day 21 by the number of inoculated cells (day 0).

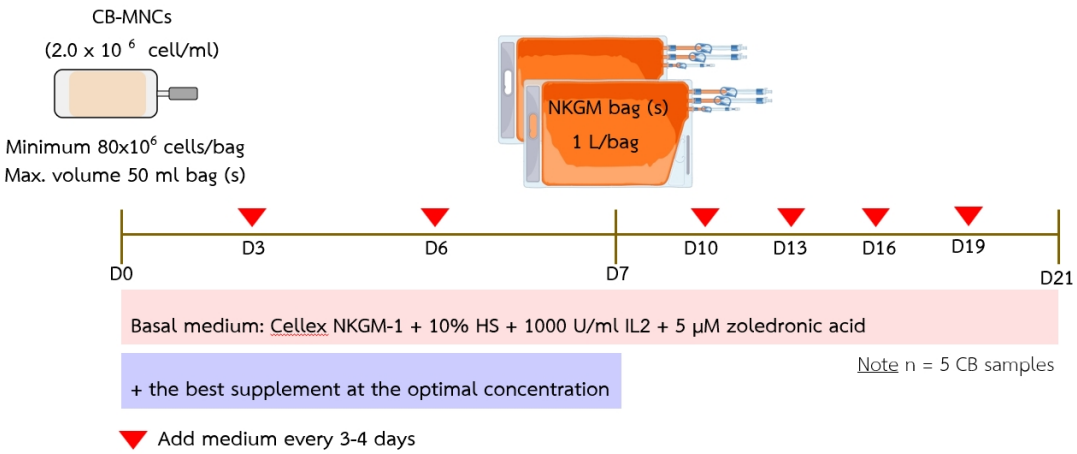

Figure 3.2 The schematic diagram of the upscaling expansion experiment.

3.3.6 Real-time PCR

Total RNAs were extracted using an easy-spin total RNA extraction kit (iNtRON Biotechnology). On-column DNase I (Invitrogen) treatment were performed at 37°C for 30 min during RNA extraction. 1 ug of total RNA was reverse-transcribed

to cDNA using Maxime RT PreMix Kit (Intron Biotech). 20 µl of PCR reaction mixture were contained 20 ng cDNA template, 10 µl of 2x KAPA SYBR FAST qPCR master mix (Kapa Biosystems), 200 nM of forward and reverse primers, and PCR-grade water. The following cycling condition were performed on a CFX96™ real-time PCR cycler (Biorad): initial enzyme activation at 95°C for 3 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 35 sec. Melting-curve analysis were performed to verify the amplification specificity. *GAPDH* were served as an internal control of each reaction well. The relative expression of the gene was calculated using the $^{\Delta\Delta}$Ct method (Livak & Schmittgen, 2001). The human primer sets used were as follows: *GAPDH* (5'-TCAAGGCTGAGAACGGGAAG-3' and 5'-CGCCCCACTTGATTTTGGAG-3') (Dahn *et al.*, 2021); Primerbank ID: 23238195c1, *IL-2Rβ* (5'-CAGCGGTGAATGGCACTTC-3' and 5'-GGCATGGACTTGGCAGGAA-3').

3.3.7 Immunophenotyping

Lymphocyte subpopulations were analyzed using CytoFLEX flow cytometer (Beckman Coulter). Cells were stained with fluorochrome-conjugated monoclonal antibodies against human cluster of differentiation CD45-FITC, CD3-PC5.5, CD56-APC/Cy7, CD16-PE, CD314-PE (NKG2D), CD159a-APC (NKG2A), CD337-PE/Dazzle 594 (NKp30), and CD336-APC (NKp44) (all from Biolegend). Cells stained with isotype-matched antibodies were used as control. A minimum of 10,000 events were acquired and data were analyzed by CytExpert software 2.0 (Beckman Coulter).

3.3.8 NK cell cytotoxicity assay

3.3.8.1 Preparation of target cells

The cytotoxic activities of expanded cells were tested against different types of cancer cell models: chronic myeloid leukemia (K562 cells), pancreatic carcinoma (MIA PaCa-2 cells), and ovarian carcinoma (SKOV3 cells). K562, MIA PaCa-2, and SKOV3 cells were purchased from American type culture collection (ATCC). Target cells were grown following ATCC's guidelines and were transfected with constructs harboring tandem dimer Tomato (tdTomato) gene (gift from Dr. Patompon Wongtrakoongate, Faculty of Science, Mahidol University, Bankkok) using Lipofectamine 3000 according to manufacturer's recommendations (Invitrogen).

Fluorochrome-tagged target cells were then maintained in the culture condition containing 1 µg/ml puromycin (Invitrogen) until cytotoxicity assay.

3.3.8.2 Flow cytometry-based NK cytotoxicity assay

Before assay K562-tdTomato-tagged cells (or target cells) were washed 2 times by PBS. Then, the cells were counted and a total of 100 µl of cells (50,000 cells/well) were added into 96-well U-bottom plate containing 100 µl of effector cells at different effector to target (E:T) ratios ranging from 50:1, 25:1, 12.5:1, 6.25:1, 3.125:1, and 0:1. After co-incubation at 37°C in 5% CO_2 for 4 hours, the plates were centrifuged and the supernatants were then collected and stored at -80 °C for later use to measure interferon gamma (IFN-γ) production. Mixture of the cells were resuspended with 200 µl PBS containing 150 nM SYTOX® Green live/dead nucleic acid staining (Invitrogen). Cells were incubated at 37°C for 20 min in the dark. The percentage of dead target were determined by Cytoflex flow cytometer (Beckman Coulter). At least 1,000 events in the target cell gate were acquired. Cytotoxicity was expressed as the percentage of cell death within the tdTomato positive population. Specific lysis was calculated by subtracting the percentage of death target cells with the percentage of spontaneous death target cells (refer to E:T ratio = 0:1 or targets alone control).

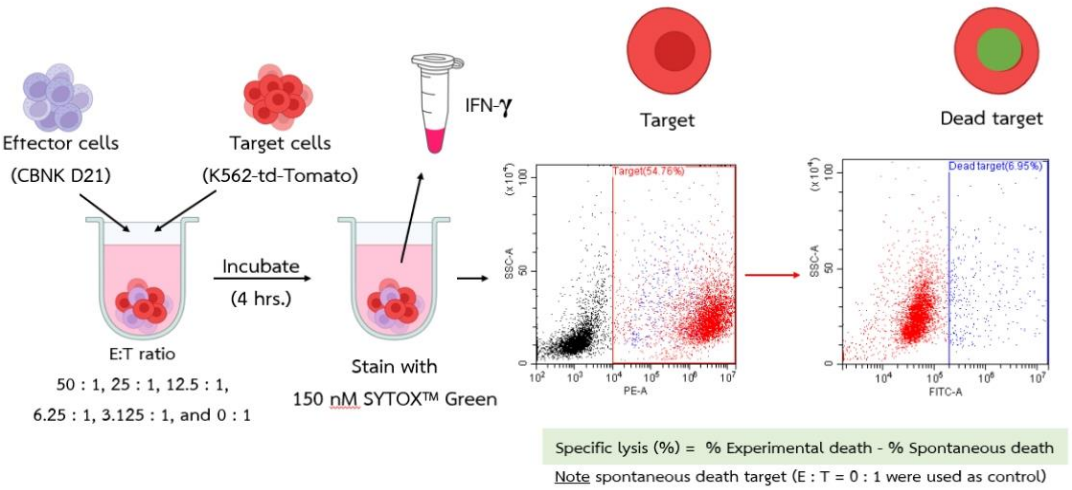

Figure 3.3 The schematic diagram of the flow cytometry-based NK cytotoxicity assay.

3.3.8.3 Microscopic-based NK cytotoxicity assay

Over night before assay, MIA PaCa-2- and SKOV3-tdTomato-tagged cells (or target cells) were plated onto 24-well plates at a seeding density of 3×10^4 cells/well. In the following day, target cells were wash 2 times by PBS and incubated with 500 µl of effector cells at 50:1, 25:1, 12.5:1, 6.25:1, 3.125:1, and 0:1 E:T ratios. After co-incubation at 37°C in 5% CO_2 for 4 hours, supernatants were collected and stored at -80°C for later use. The cells were then wash 2 times by PBS and fixed with 4% paraformaldehyde for 20 min. After washing, cell nuclei were stained with 5 µg/ml Hoechst 33342 (Invitrogen) for 10 min. After 2 times washing by PBS, the number of the remaining targets (tdTomato positive cells) were determined under a phase contrast fluorescence microscope at 10x magnification. At least 2 different fields of each sample were captured and counted. The percentage of specific lysis was calculated using the following formulation.

$$\text{Specific lysis } (\%) = 100 - \left(\frac{\text{Number of target cells experiment}}{\text{Number of target cells spontaneous}} \times 100 \right)$$

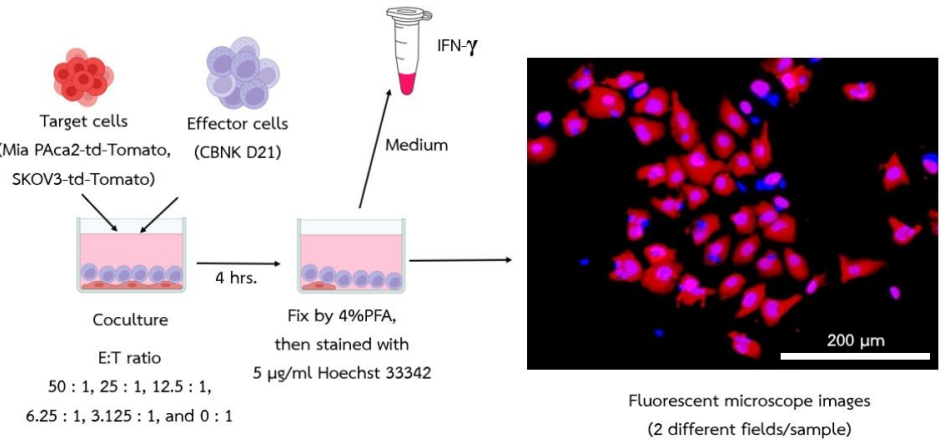

Figure 3.4 The schematic diagram of the microscopic-based NK cytotoxicity assay.

3.3.9 Interferon-gamma (IFN-γ) release assay

Production of IFN-γ in the cell culture supernatants during NK cytotoxicity test of 50:1 (as maximum release) and 0:1 (as negative control) E:T ratios

were measured by the NK Vue® ELISA kit (NKMax, Seongnam-si, Korea) in accordance with the manufacturer's protocol. Absorbance was measured at 450 nm using Azure Biosystems (Dublin, CA, USA) microplate reader.

3.3.10 Statistical Analysis

Data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.). Data comparison were made using one-way ANOVA with post hoc Tukey's tests and paired, two-tailed t-tests. A p-value of less than 0.05 was considered statistically significant.

3.4 Results

3.4.1 LPS promote the proliferation of CBNK cells

To obtain high NK cell numbers, we first investigated the effect of LPS and Yhhu-3792 on CBNK cell expansion. In this experiment, the chosen concentration of LPS (1 µg/mL) and Yhhu-3792 (2.5 µM) were first based on the previous finding from PBMCs and neural stem cells (Goodier *et al.*, 2000; Lu *et al.*, 2018). After density gradient separation, CB-MNCs, with an average $CD56^{+}CD3^{-}$ NK cell purity of 2.85±0.87% (n = 5), were cultured in Cellex NKGM-1 medium supplemented with cytokines and were allowed to expanded in the absence or presence of LPS or Yhhu-3792 for 7 days. After day 7, cells were cultured for a further 7 days to allow the cells to acquire a mature phenotype. Over a 14-day culture period, it was observed that cells grew as floating cell aggregates, with no morphological differences observed between groups (Figure 3.5). On day 7 and day 14, results from flow cytometry analysis showed that the percentage of $CD56^{+}CD3^{-}$ NK cells were significantly higher in cultures containing LPS (D7: 64.48±8.45% and D14: 80.06±9.35%) compared to Yhhu-3792 (D7: 40.36±9.46% and D14: 49.00±16.58%) and control (D7: 40.66±8.40% and D14: 50.90±19.27%) groups (Figure 3.6A and 3.6B).

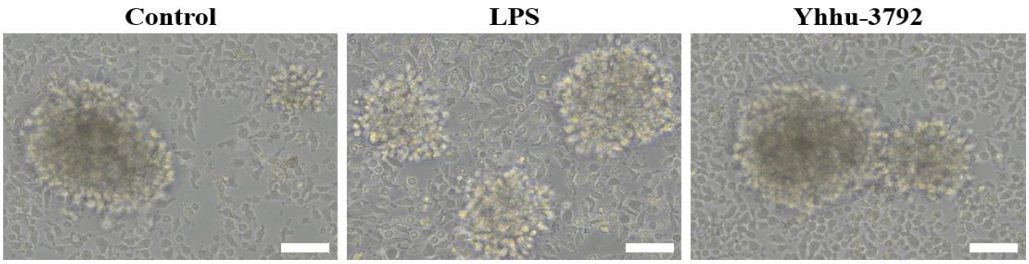

Figure 3.5 Morphology of the expanded cells at day 14. The bar represents 50 µm, corresponding to 20x magnification.

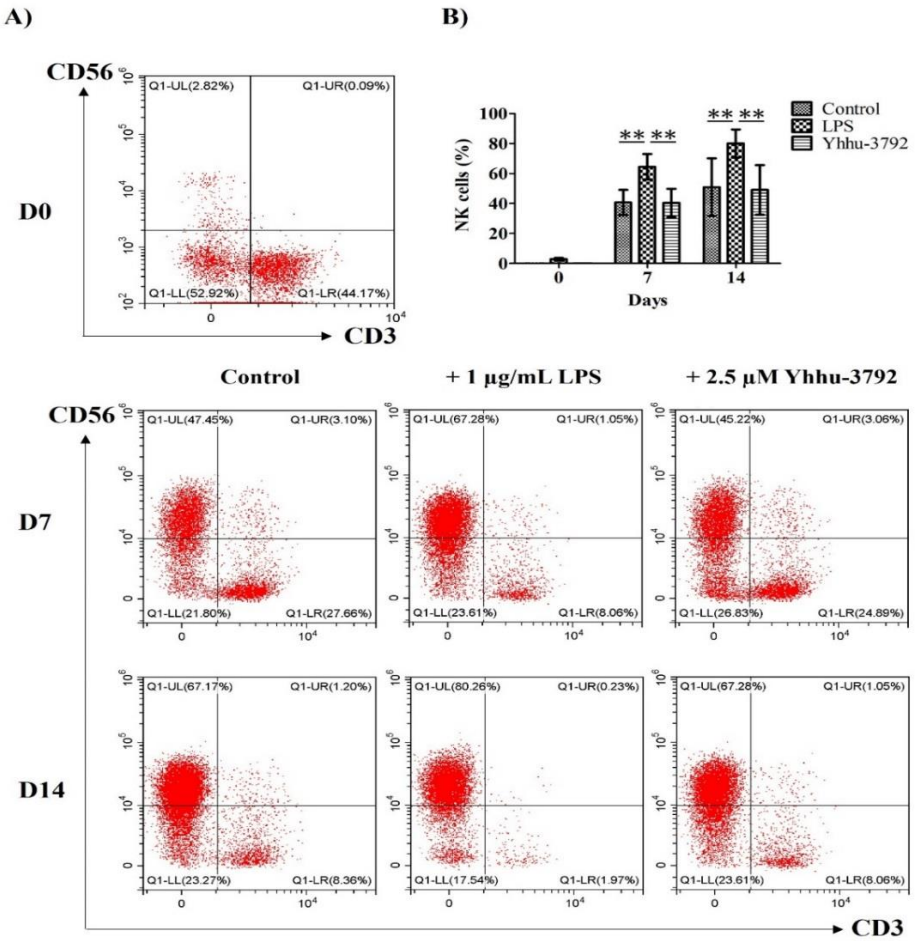

Figure 3.6 The effect of LPS and YHHU on proportion of CBNK cells. A) Representative flow cytometry dot plots of $CD56^{+}CD3^{-}$ NK cells on day 0, day 7, and day 14 under LPS and YHHU stimulation. B) The percentage of $CD56^{+}CD3^{-}$ NK cells growth under tested conditions. Data are expressed as the mean ± SEM (n = 5).

Of note, it was worth mentioning that the expansion of $CD56^{+}CD3^{-}$ NK cells in LPS group were negatively associated with the frequency of $CD3^{+}$ T cells. In LPS group, the percentage of $CD3^{+}$ T cells gradually declined over the 14-day expansion period, decreasing from 49.97±5.06% on day 0 to 20.70±16.45% on day 7, and to 15.92±9.98% on day 14 (Figure 3.7). Since the addition of LPS (1 μg/mL) resulted in the highest NK cell purity, all subsequent experiments were conducted using LPS supplementation.

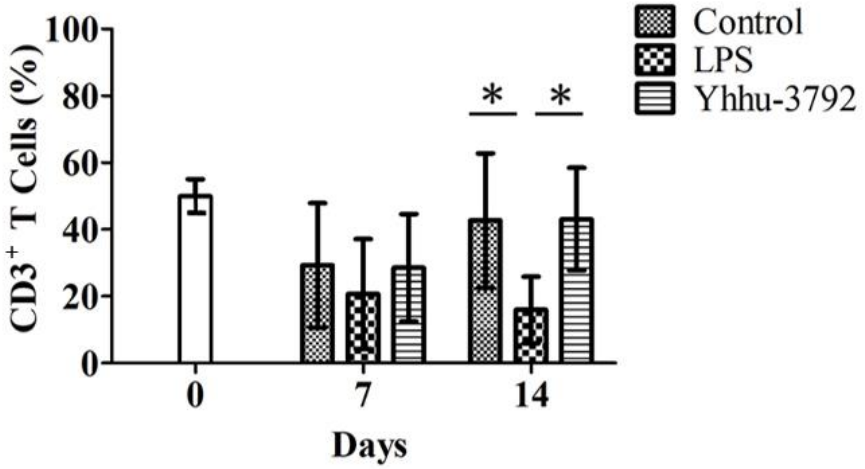

Figure 3.7 The percentage of $CD3^{+}$ T cells over the 14-day expansion. Data are expressed as the mean ± SEM

3.4.2 Effect of LPS concentration on CBNK cell expansion

To ensure that the use of LPS stimulation caused no adverse effects on CBNK cells, we first checked a safe range of concentrations before the optimization step took place. Results from the CCK-8 assay show that a difference in cell toxicity occurs in the group treated with 20 ug/ml LPS (figure 3.8). This study therefore narrows down the tested concentration to 0, 0.1, 1.0, 5.0, and 10 μg/mL. As shown in Figure 3.9, high percentage of $CD56^{+}CD3^{-}$ NK cells were obtained in the groups at or above 1 μg/mL of LPS (n = 5). The percentage of $CD56^{+}CD3^{-}$ NK cells on day 7 and day 14 were as follows: 0 μg/mL (D7: 36.58±5.49% and D14: 66.41±16.23%), 0.1 μg/mL (D7: 39.46±5.49% and D14: 67.85±17.50%), 1.0 μg/mL (D7: 56.87±4.92% and D14: 85.21±10.24%), 5.0 μg/mL (D7: 56.14±4.64% and D14: 86.41±7.72%), and 10 μg/mL (D7: 55.01±5.97 % and D14:

80.40±14.55%). As we did not observe any significant difference when the concentration of LPS was greater than 1 μg/mL, so the concentration of LPS at 1 μg/mL was used for upscaling production of CBNK cells.

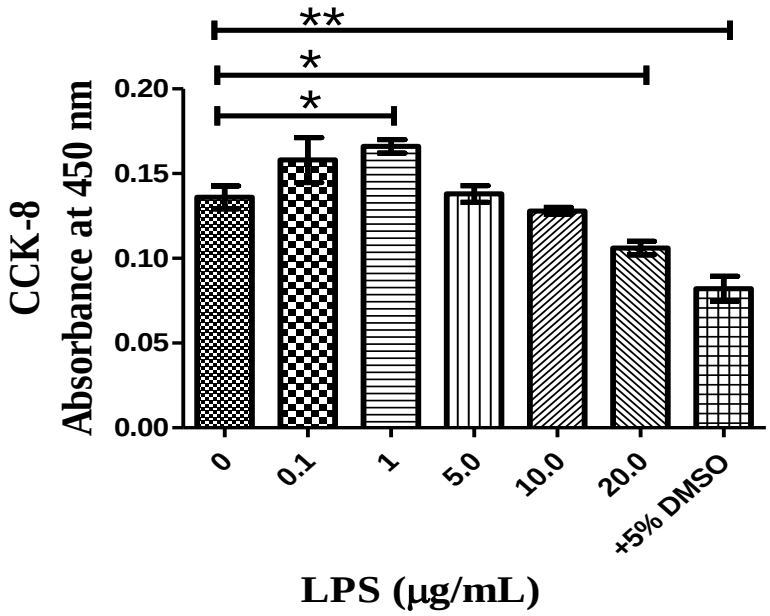

Figure 3.8 The effect of LPS concentration on CCK-8 response. Data are expressed as the mean ± SEM of OD values.

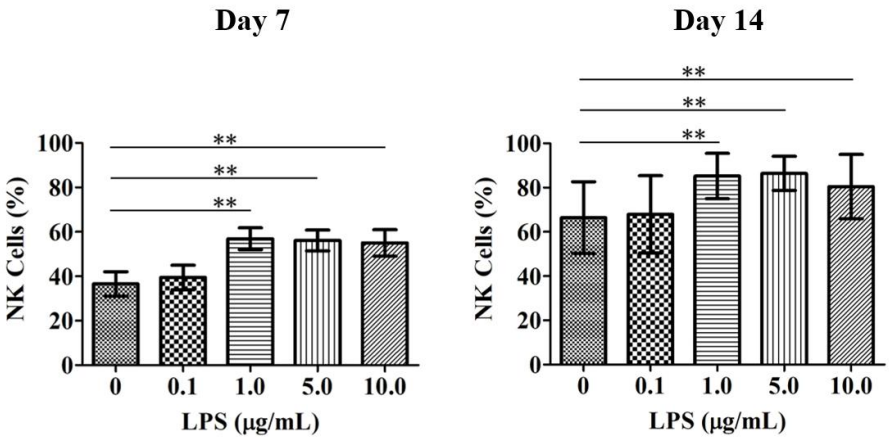

Figure 3.9 The percentage of NK cells in different LPS concentrations. Data are expressed as the mean ± SEM (n = 5).

3.4.3 Upscaling production of CBNK cells

To further investigate the maximum expansion capability of CBNK cells using the LPS stimulation approach, this study proceeded by cultivating CB-MNCs (n = 5) in a cell culture bag in the presence of 1 µg/mL LPS for 7 days. Subsequently, the activated cells were transferred to two CO_2-permeable culture bags (1 L medium/bag) and then cultured for an additional 14 days with the replenishment of fresh medium every 3 days. After 21 days, this culture system yielded an average of 16,800.00±2,900.00×10^6 total nucleated cells (TNCs) starting from an average of 110.94±28.52×10^6 CB-MNCs (Figure 3.10A). On day 21, the average purity of $CD56^+CD3^-$ NK cells and $CD3^+$ T cells is 92.09±3.47% and 2.70±1.14%, respectively. The dynamics of $CD56^+CD3^-$ NK cells and $CD3^+$ T cells throughout the expansion period were shown in Figure 3.10B. Of note, it was clearly seen that the population of $CD56^+CD3^-$ NK cells dominated the culture during the first week of expansion. This finding was in accordance with a significant upregulation of an *interleukin-2 receptor subunit beta* (*IL2Rβ*) gene. In humans, expression of *IL2Rβ* marks an irreversible commitment towards NK lineage of common lymphoid progenitor (CLP) cells (Abel *et al.*, 2018). Compared to unexpanded CB-MNCs, the expression of *IL2Rβ* increased by 40 times and 149 times on day 7 and day 21, respectively (Figure 3.11).

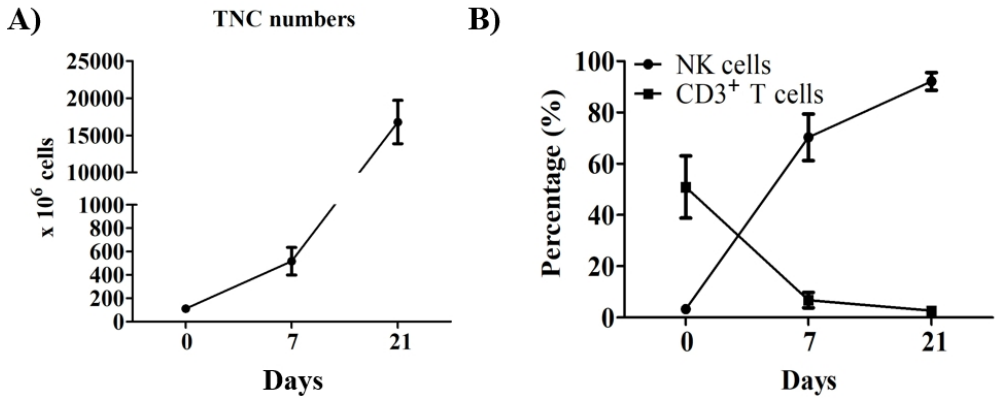

Figure 3.10 Upscaling expansion of NK cells using the LPS stimulation approach. A) Average yield of TNCs expansion and (B) the proportion of NK and T cells on day 0, day 7, and day 21. Data are expressed as the mean ± SEM (n = 5).

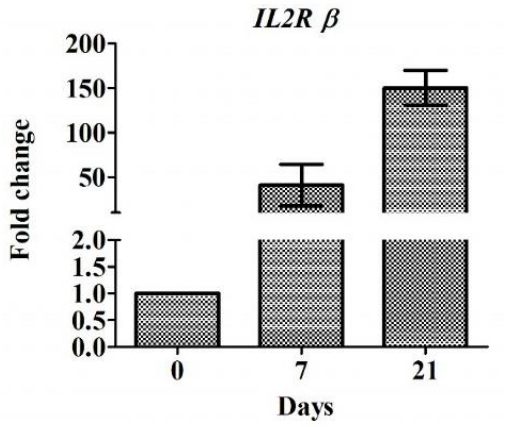

Figure 3.11 The gene expression levels of *IL2Rβ*. The expression values were normalized to *GAPDH*. Data are expressed as the mean ± SEM.

To understand the phenotypes of the expanded cells (day 21), an in-depth flow cytometry analysis was employed using a set of known NK cell-associated markers; activating receptors (CD16 and NKG2D), inhibitory receptor (NKG2A), and natural cytotoxicity receptor (NKp30 and NKp44). In human NK cells, the expression of NKG2A, natural cytotoxicity receptor, and activating receptors indicates the maturation status of the cells (Abel *et al.*, 2018). The results from the flow cytometry analysis demonstrate that the population of $CD56^{+}CD3^{-}$ NK cells co-expressed CD16 (83.63±8.27%), NKG2D (98.33±1.55%), NKG2A (73.92±12.09%), NKp30 (73.42±17.55%), and NKp44 (36.74± 11.12%) (figure 3.12A and 3.12B). These data demonstrate that CBNK cells produced by this approach are phenotypically mature NK cells.

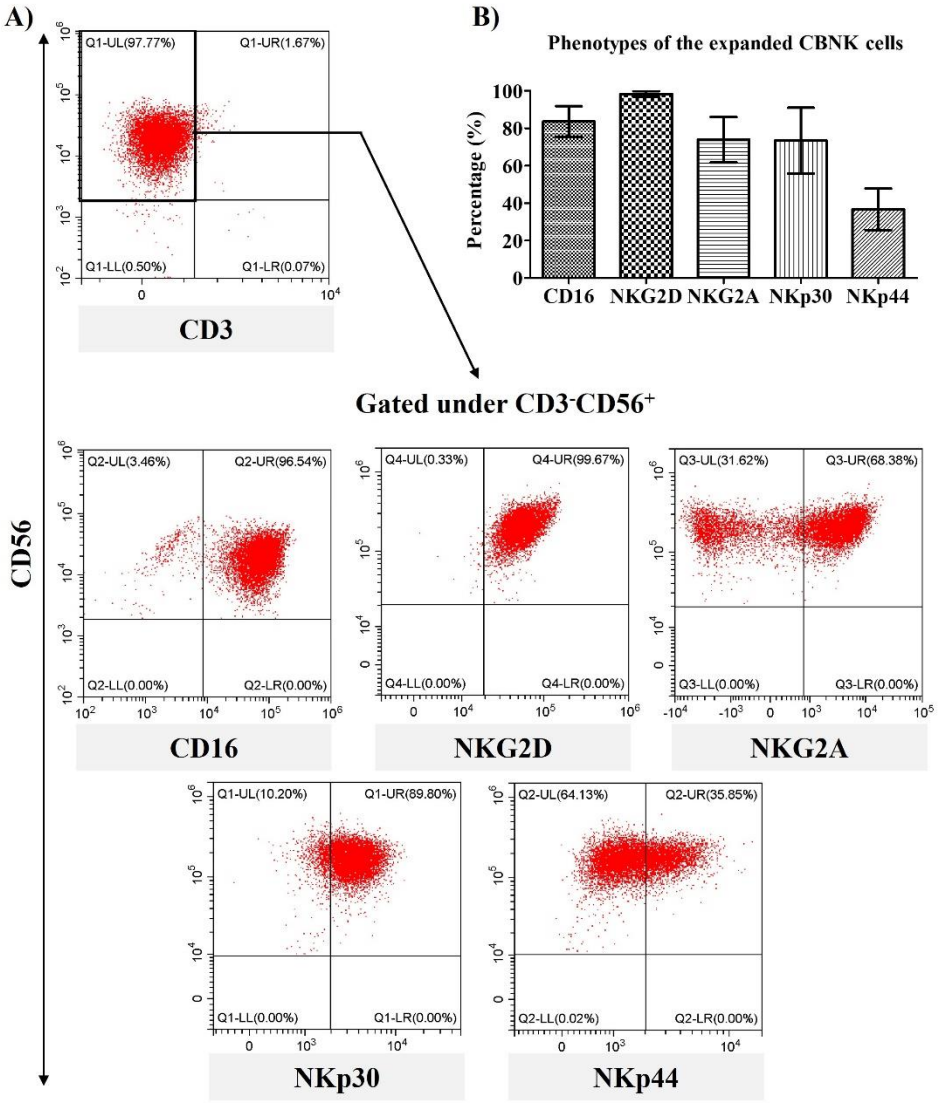

Figure 3.12 Immunophenotype profile of the expanded cells. A) Representative flow cytometry dot plots of the expanded cells (day 21). B) The percentage of NK cell-associated markers on the expanded cells (day 21). Data are expressed as the mean ± SEM (n = 5).

3.4.4 Anti-cancer activities of expanded CBNK cells.

One important role of NK cells in the immune response is to identify and destroy abnormal cells, such as cancer cells, through missing-self and induced-self recognition mechanisms (Barrow *et al.*, 2019). To investigate whether the expanded CBNK cells acquire this role, this study next performed cytotoxicity tests against three

distinct cancer cell models: chronic myeloid leukemia (K562 cells), pancreatic carcinoma (MIA PaCa-2 cells), and ovarian carcinoma (SKOV3 cells). After 4 hours of co-incubation, the expanded CBNK cells showed high level of cytotoxicity against K562 (figure 3.13), MIA PaCa-2 (figure 3.14), and SKOV3 cells (figure 3.15).

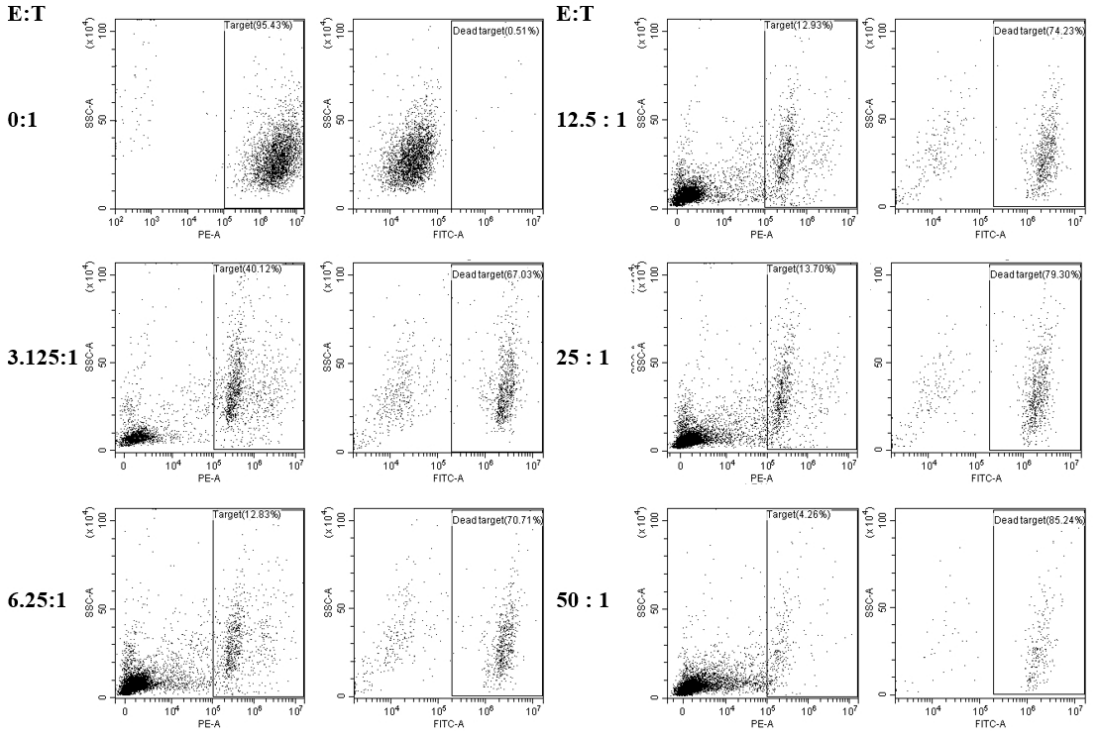

Figure 3.13 Cytotoxicity of the expanded CBNK cells against K562 tdTomato-tagged cells. Representative dot plot showing the initial gate (left; PE-A/SSC-A plot) and subsequent target cell plot (right; FITC-A/SSC-A plot). Dead targets were FITC-positive cells gated from the PE-positive population.

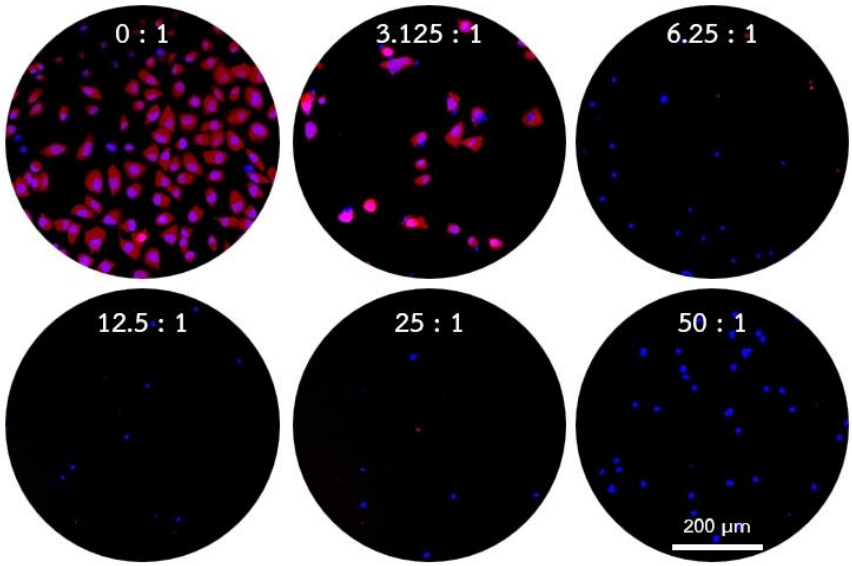

Figure 3.14 Cytotoxicity of the expanded CBNK cells against MIA PaCa-2 tdTomato-tagged cells. Representative fluorescence microscopic image of MIA PaCa-2 cells (red) after 4 hours of co-incubation with expanded CBNK cells (n = 5). The bar represents 200 µm, corresponding to 10x magnification.

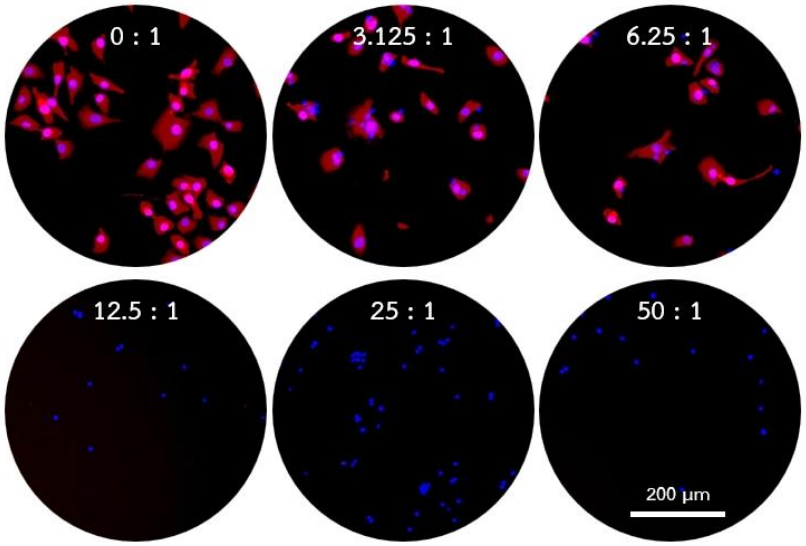

Figure 3.15 Cytotoxicity of the expanded CBNK cells against SKOV3 tdTomato-tagged cells. Representative fluorescence microscopic image of SKOV3 cells (red) after 4 hours of co-incubation with expanded CBNK cells (n = 5). The bar represents 200 µm, corresponding to 10x magnification.

As shown in Figure 3.16A, the percentage of specific lysis increases in an E:T ratio-dependent manner. Of note, more than 50% specific lysis was observed at E:T ratios as low as 3.125:1 in all tested models. Furthermore, to better understand how expanded CBNK cells mediate cancer killing, this study next measured IFN-γ secretion during cytotoxicity testing. As shown in Figure 3.16B, high levels of IFN-γ were detected in the co-culture supernatants of 50:1 ratio, but not in 0:1 ratio, in all tested cancer cell models. Collectively, these results demonstrate that the expanded CBNK cells are functional cells.

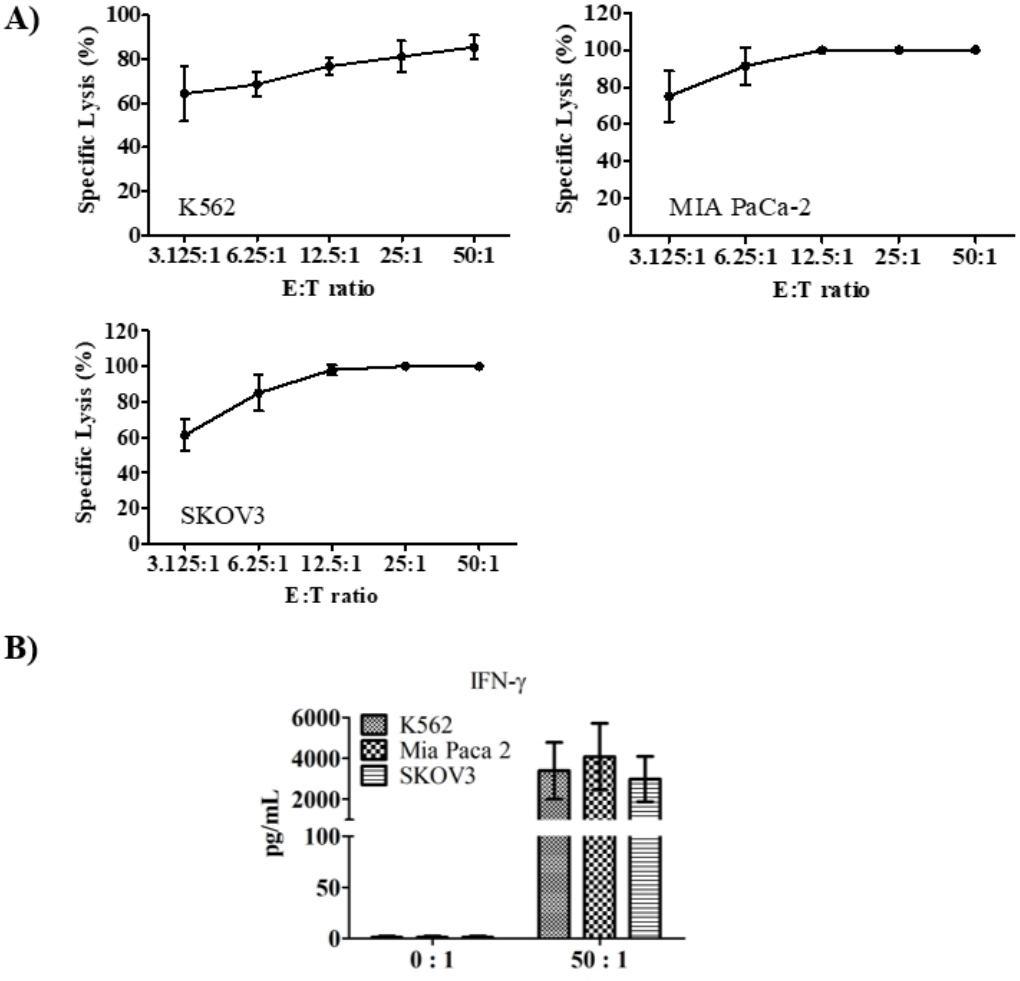

Figure 3.16 Anti-cancer activities of expanded CBNK cells. A) NK cell cytotoxicity tests against K562, MIA PaCa-2, and SKOV3 cells. B) IFN-γ secretion in co-culture media during cytotoxicity testing. Data are expressed as the mean ± SEM (n = 5).

3.5 Discussion

Recently, adoptive transfer of allogeneic NK cells has gained attention as a safe and effective treatment for various types of cancer. This approach involves infusing expanded NK cells from a healthy donor into the patient, where they can target and eliminate cancer cells without causing any significant side effects (Lim *et al.*, 2015). Typically, the number of NK cells used in clinical trials ranges from 5×10^6 to 1×10^8 cells per kilogram of body weight (Heipertz *et al.*, 2021). With the rapid advancement of biotechnology, it has now become feasible to expand these clinically relevant doses of NK cells in the laboratory (Fang *et al.*, 2022). While it is possible to expand NK cells from various sources (including peripheral blood, human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs), and cell lines such as NK-92 cells), it is reasonable to infer that umbilical cord blood (CB) is the most promising source due to its safe and limitless supply.

To obtain large numbers of CBNK cells, feeder cells and cell sorting processes have been used in several studies. For instance, the use of Epstein-Barr Virus transformed lymphoblastoid cell lines (EBV-LCL), pioneered by Dr. Richard Childs and colleagues at the National Institutes of Health (Igarashi *et al.*, 2004), together with low concentrations of IL-2 (100 IU/mL) and IL-15 (5 ng/mL) generated an average 700-fold expansion of CBNK cells, with a purity of 94.28±2.08%, from CD3-depleted CB-MNCs (Reina-Ortiz *et al.*, 2020). Besides tumor cell lines, Hosseini *et al*. demonstrated that coculturing CD3-depleted CB-MNCs with Mitomycin C-treated bone marrow stem cells in the presence of IL-2, IL-15, IL-3, and Fms-related tyrosine kinase 3 ligand (FLT-3L) for 2 weeks can result in an average 104±15-fold expansion with a purity of 65±10% of CBNK cells (Hosseini *et al.*, 2017). However, although a substantial number of CBNK cells can be attained using these feeder cell techniques, it's important to note that there are safety concerns related to the persistence of feeder cells or the contamination of feeder-derived impurities in the final product. Additionally, while offering a potential method to enrich specific cell populations, cell sorting comes with certain drawbacks including the potential for cell loss, induction of cell stress, and a costly and time-consuming process. This study, therefore, offers a feeder-free and cell-sorting-free approach for expanding purified CBNK cells.

As a way to generate high numbers of CBNK cells, directing NK cell development in the early stages of expansion is crucial. Experimental evidence suggests that Notch signaling plays different roles during NK cell development (Felices *et al.*, 2014). Previous studies have shown that cell fate decisions of human $CD34^+$ HSCs are tightly regulated by the Notch pathway; low and high Notch activation resulted in an increase in the frequency of NK and T cell precursors, respectively (Benne *et al.*, 2009). Recently, it has been shown that a small molecular compound of Notch agonist can be used to promote the proliferation and activation of PBNK cells (Dongdong *et al.*, 2019). In a different approach that could lead to the production of purified CBNK cells, LPS has been shown to enhance the proliferation and induced activation of human PBNK cells. By tracing peripheral blood-derived mononuclear cells (PB-MNCs) with carboxyfluorescein succinimidyl ester (CFSE) dye, Goodier & Londei demonstrate that $CD56^+$ NK cells, but not $CD3^+$ T cells, predominantly proliferate under LPS treatment; the mean percentage of $CD56^+$ NK cells before and after stimulation is 10.5±3.5% and 20.9±11.3%, respectively (Goodier *et al.*, 2000).

By translating these findings into CBNK cell production, this study first uses Yhhu-3792, and LPS to stimulate CBNK cell proliferation during the first week of expansion. On one hand, the immunophenotyping results of day 7 and day 14 samples demonstrate that the percentage of $CD56^+CD3^-$ NK cells was significantly ($P<0.01$) higher in the LPS group compared to the Yhhu-3792 and control groups. This finding further reveals the effect of LPS on the proliferation of CBNK cells. On the other hand, there was no considerable difference in the percentage of $CD56^+CD3^-$ NK cells observed between the Yhhu-3792 and control groups. The hypothesis was that this is due to the distinct characteristics (i.e., subpopulation distribution) of the starting samples or the potential differences in the development pathways between PBNK and CBNK cells. Further research on Notch agonist and CBNK cell development is encouraged.

After optimizing the LPS concentration, the upscaling experiment in the cell culture bag revealed that the proposed procedure yielded up to 15,400.00± 2,600.00×10^6 $CD56^+CD3^-$ NK cells, with an average of 2.70±1.14% of $CD3^+$ T cells. As previously mentioned, this quantity of CBNK cells is sufficient for performing ACT in adulthood. Previously, it has been reported that CBNK cells have an immature

phenotype and are functionally deficient (Gaddy *et al.*, 1995; Luevano *et al.*, 2012). Therefore, to ensure the desirable outcome in cancer treatment, it is worth showing that the expanded CBNK cells are mature NK cells with cancer-killing activities. As shown in figure 3.12, CBNK cells produced by this procedure highly expressed several NK cell-associated receptors, including activating receptors (CD16 and NKG2D), inhibitory receptor (NKG2A), and natural cytotoxicity receptors (NKp30 and NKp44). Consistent with this finding, cytotoxicity test results demonstrate that the expanded CBNK cells possess cytolytic activity as well as the capability to secrete inflammatory cytokine IFN-γ, enabling them to target and destroy various types of cancer cell models used in this study.

The roles of LPS in stimulating immune cells have been reported elsewhere (Lawlor *et al.*, 2021; McAleer *et al.*, 2008). This study has provided an additional insight of LPS on CBNK cell proliferation. In the vaccine industry, LPS and modified LPS are considered as potent adjuvants (Zariri *et al.*, 2015). However, it should bear in mind that its powerful biological activities are associated with toxicity. This study, therefore, takes advantage of the developmental opportunity of CB-MNCs by applying LPS for the first 7 days of expansion solely to direct NK cell development, then withdrawing. Based on the up-regulation of *IL2Rβ* (figure 3.11) and the lack of alteration in the proliferative trend of the expanded CBNK cells upon treatment removal, it is possible that NK lineage commitment occurred during the stimulation period.

3.6 Conclusions

This study has developed a feeder-free and cell sorting-free protocol for the expansion of purified CBNK cells with intact cancer-killing capability. Translating this simple, but reliable, technology into manufacturing scale production will not only light up new opportunities in cancer treatment but also provide expansion platform for next-generation NK cells. Chimeric antigen receptor NK cells, bi- and tri-specific killer engagers, as well as NK cell-derived exosomes, are upcoming technologies that are being developed to fight against a diverse range of cancers.

3.7 References

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