EFFECTS OF LPS AND YHHU-3792 ON THE EXPANSION OF NATURAL KILLER CELLS DERIVED FROM HUMAN UMBILICAL CORD BLOOD

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ผลของ LPS และ Yhhu-3792 ต่อการเพิ่มจำนวนเซลล์เม็ดเลือดขาวชนิด เอ็น เค จากเลือดสายสะดือมนุษย์

นางหทัยวรรณ กุลกาญจนาวรรณ

วิทยานิพนธ์นี้สำหรับการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy.

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คำสำคัญ : ภูมิคุ้มกันบำบัดรักษามะเร็ง/เซลล์เม็ดเลือดขาวชนิด เอ็น เค/เลือดสายสะดือ/เซลล์เม็ดเลือดขาวชนิด เอ็น เค จากเลือดสายสะดือ

การปลูกถ่ายเซลล์เม็ดเลือดขาวชนิด เอ็น เค ที่ผ่านการเพิ่มจำนวนแล้วถือเป็นความหวังสำคัญของภูมิคุ้มกันบำบัดรักษามะเร็ง อย่างไรก็ตามยังมีหลายปัจจัยที่ต้องคำนึงถึงเมื่อต้องการใช้เซลล์เม็ดเลือดขาวชนิด เอ็น เค ในการรักษามะเร็ง เช่น แหล่งที่มาของเซลล์เม็ดเลือดขาวชนิด เอ็น เค สภาวะที่ใช้ในการเพาะเลี้ยงเพิ่มจำนวนเซลล์ จำนวนและความบริสุทธิ์ของเซลล์เม็ดเลือดขาวชนิด เอ็น เค ภายหลังการเพาะเลี้ยง และ ความสามารถในการทำลายเซลล์มะเร็งของเซลล์ที่ผ่านการเพาะเลี้ยง เพื่อสร้างแหล่งที่มาที่ยั่งยืนของเซลล์เม็ดเลือดขาวชนิด เอ็น เค เพื่อการใช้ประโยชน์ทางคลินิก การศึกษานี้ได้พัฒนาวิธี feeder-free และ cell-sorting-free เพื่อเพิ่มจำนวนเซลล์เม็ดเลือดขาวชนิด เอ็น เค จากเลือดสายสะดือ โดยเมื่อกระตุ้นเซลล์ชนิด mononuclear ที่คัดแยกได้จากเลือดจากสายสะดือ (cord blood derived mononuclear cells) ด้วย Lipopolysaccharide (LPS ความเข้มข้น 1 ไมโครกรัม/มิลลิลิตร) ในช่วงสัปดาห์แรกของการเพาะเลี้ยง การศึกษานี้แสดงให้เห็นว่าการเติม LPS สามารถกระตุ้นการเพิ่มจำนวนของเซลล์เม็ดเลือดขาวชนิด เอ็น เค ได้อย่างมีนัยสำคัญทางสถิติ ($P<0.01$) ทั้งนี้เมื่อขยายระดับการผลิตขึ้นพบว่าวิธีการที่พัฒนาขึ้นในการศึกษานี้สามารถเพิ่มจำนวนเซลล์ได้เฉลี่ย $1.68\pm2.92\times10^{10}$ เซลล์จากเลือดสายสะดือ 1 ยูนิต โดยพบว่าร้อยละ 92.09±3.47 ของเซลล์ที่เพิ่มจำนวนได้คือเซลล์เม็ดเลือดขาวชนิด เอ็น เค ผลการวิเคราะห์ด้วยเทคนิค flow cytometry แสดงให้เห็นว่าเซลล์เม็ดเลือดขาวชนิด เอ็น เค ที่เพาะเลี้ยงได้มีการแสดงออกร่วมของ marker ต่าง ๆ ของเซลล์เม็ดเลือดขาวชนิด เอ็น เค ได้แก่ CD16 (ร้อยละ 83.63±8.2), NKG2D (ร้อยละ 98.33±1.55), NKp30 (ร้อยละ 73.42±17.55), NKp44 (ร้อยละ 36.74±11.12) และ NKG2A (ร้อยละ 73.92±12.09) นอกจากนั้นการทดสอบฤทธิ์การทำลายเซลล์มะเร็งของเซลล์เม็ดเลือดขาวชนิด เอ็น เค ที่เพิ่มจำนวนได้ในแบบจำลองเซลล์มะเร็ง ชนิด K562 (chronic myeloid leukemia cells), MIA PaCa-2 (pancreatic carcinoma cells) และ SKOV3 (ovarian carcinoma cells) แสดงให้เห็นอัตราการตายของเซลล์มะเร็งมากกว่าร้อยละ 50 ในทุกๆ ชนิดของเซลล์มะเร็งที่ทดสอบ (ณ อัตราส่วน effector ต่อ target ที่ 3.125 ต่อ 1)

การทดลองครั้งนี้สรุปได้ว่าวิธีการเพาะเลี้ยงเซลล์แบบ feeder-free และ cell-sorting-free ที่พัฒนาขึ้นในการศึกษานี้สามารถเพิ่มจำนวนเซลล์เม็ดเลือดขาวชนิด เอ็น เค จากเลือดสายสะดือได้

อย่างมีประสิทธิภาพ โดยเซลล์ที่เพาะเลี้ยงได้จากวิธีการดังกล่าวมีความบริสุทธิ์ของเซลล์เม็ดเลือดขาวชนิด เอ็น เค, มีจำนวนเซลล์ที่เพียงพอต่อการนำไปใช้ทางคลินิก และยังคงไว้ซึ่งการทำงานที่สมบูรณ์ของเซลล์

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____

HATAIWAN KUNKANJANAWAN : EFFECTS OF LPS AND YHHU-3792 ON THE EXPANSION OF NATURAL KILLER CELLS DERIVED FROM HUMAN UMBILICAL CORD BLOOD. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 58 PP.

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Adoptive cell transfer of *ex vivo* expanded natural killer (NK) cells holds significant hope in cancer immunotherapy. However, some points of using NK cells in cancer treatment include source of NK cells, conditions for their expansion, resulting number and purity of NK cells after culturing, and anti-cancer activity of the expanded cells need to be considered. To establish a sustainable supply of NK cells for clinical applications, this study has developed a feeder-free and cell sorting-free method for expanding cord blood-derived NK (CBNK) cells. By stimulating cord blood derived mononuclear cells (CB-MNCs) with Lipopolysaccharide (LPS, 1 ug/mL) during the first week of expansion, this study demonstrates that the presence of LPS triggered a significant increase in CBNK cell proportions (P<0.01). Further upscaling of the expansion reveals that, from a single cord blood unit, the proposed procedure results in an average of $1.68\pm2.92\times10^{10}$ total nucleated cells (TNCs), of which 92.09±3.47% of the expanded cells are NK cells. Results from flow cytometry analysis showed that the expanded CBNK cells were co-positive with various NK cell markers: CD16 (83.63±8.27%), NKG2D (98.33±1.55%), NKp30 (73.42±17.55%), NKp44 (36.74±11.12%), and NKG2A (73.92±12.09%). Moreover, the anti-cancer activities of the expanded CBNK cells were investigated in K562, MIA PaCa-2, and SKOV3 cells; each serving as a representative cancer cell model for chronic myeloid leukemia, pancreatic carcinoma, and ovarian carcinoma, respectively. After 4 hours of co-incubation, more than 50% specific lysis was observed at effector to target cell ratios as low as 3.125:1 in all tested models. Collectively, these results illustrate that the proposed feeder-free and cell-sorting-free expansion method can effectively yield clinically relevant doses of purified CBNK cells with intact functions.

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Hataiwan Kunkanjanawan

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LIST OF ABBREVIATIONS

aAPCs	=	Artificial antigen-presenting cells
ACT	=	Adoptive cell transfer
ADCC	=	Antibody-dependent cell-mediated cytotoxicity
ALL	=	Acute lymphocytic leukemia
AML	=	Acute myeloid leukemia
ATMPs	=	Advanced therapy medicinal products
AZA	=	Azelaic acid
CAR	=	Chimeric antigen receptor
cATMPs	=	combined ATMPs
CB	=	Cord blood
CB-MNCs	=	Cord blood derived mononuclear cells
CBNK cells	=	Cord blood-derived NK cells
CFSE	=	Carboxyfluorescein succinimidyl ester
CGT	=	Cell and Gene Therapy
CLL	=	Chronic lymphocytic leukemia
CLP cells	=	Common lymphoid progenitor cells.
CPAs	=	Cryoprotectant agents
CRF	=	Controlled Rate Freezer
D	=	Dimension
DAMPs	=	Danger-Associated Molecular Patterns
DMSO	=	Dimethyl sulfoxide
DNAM-1	=	DNAX Accessory Molecule-1
EBV-LCL	=	Epstein-Barr Virus transformed lymphoblastoid cell lines
EMA	=	European medicines agency
ESCs	=	Embryonic stem cells
EU	=	European Union
FasL	=	Fas ligand

LIST OF ABBREVIATIONS (Continued)

FDA	=	Food and drug administration
FLT-3L	=	Fms-related tyrosine kinase 3 ligand
G-CSF	=	Granulocyte colony-stimulating factor
GM-CSF	=	Granulocyte macrophage colony-stimulating factor
GMP	=	Good Manufacturing Practice
GSIs	=	Gamma secretase inhibitors
GTMP	=	Gene therapy medicinal products
GvHD	=	Graft versus host disease
GZMB	=	Granzyme B
HBV	=	Hepatitis B virus
HCC	=	Hepatocellular carcinoma
HCV	=	Hepatitis C virus
HIV	=	Human immunodeficiency virus
HLA	=	Human leukocyte antigen
HSCs	=	Hematopoietic stem cells
HSCT	=	Hematopoietic stem cell transplantation
IFN-γ		Interferon-gamma
IL	=	Interleukin
IL2Rβ	=	interleukin-2 receptor subunit beta
KIR	=	Killer immunoglobulin-like receptors
LIF	=	Leukemia inhibitory factor
LPS	=	Lipopolysaccharide
mAb	=	Monoclonal antibody
mb	=	Membrane bound
MCL	=	Mantle cell lymphoma
MDS	=	Myelodysplastic syndromes
MIP-Iα	=	Macrophage inflammatory protein-1 alpha
MM	=	Multiple myeloma
ND	=	Not determined

LIST OF ABBREVIATIONS (Continued)

NHL	=	Non-hodgkin's lymphoma
NK cells	=	Natural killer cells
NKG2A	=	NK group 2 member A
NKG2D	=	Natural killer group 2 member D
PBS	=	Phosphate buffered saline
PB-MNCs	=	Peripheral blood derived mononuclear cells
PBNK cells	=	Peripheral blood derived NK cells
PRF1	=	Perforin 1
RCC	=	Renal cell carcinoma
SCF	=	Stem cell factor
SCTMP	=	Somatic cell therapy medicinal products
T cells	=	T lymphocytes
TEP	=	Tissue-engineered products
TLR4	=	Toll-like receptor 4
TPO	=	Thrombopoietin
TNF-α	=	Tumor necrosis factor-alpha
TRAIL		Tumor Necrosis Factor-related apoptosis-inducing ligand
TRIF	=	TIR domain-containing adaptor inducing IFN-β
T-SNE plot	=	T-distributed neighbor embedding plot
UCB34	=	NK cells expanded from $CD34^+$ cells
UCB56	=	NK cells expanded from $CD56^+$ cells
US	=	United States
NK cells	=	Natural killer cells

CHAPTER I

INTRODUCTION

1.1 Background and significance

In recent years, adoptive cell therapy has arisen as an attractive therapeutic option to treat advanced refractory or unresectable cancers. Natural killer cells (NK cells) are cytotoxic lymphocytes that play important roles in early defense against viral infections and tumor surveillance. Without any priming or prior sensitization, NK cells exert its cytolytic activity against viral infected cells and tumor cells via difference mechanisms: direct cytotoxicity via releasing of lytic granules containing perforin and granzymes, activation of antibody-dependent cell-mediated cytotoxicity (ADCC), induction of death receptor-mediated apoptosis via binding of Tumor Necrosis Factor-related apoptosis-inducing ligand (TRAIL) or Fas ligand (FasL) on target cells, and secreting inflammatory cytokines (interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α)) (Wang *et al.*, 2020). Moreover, compared with cytotoxic T lymphocytes (T cells), NK cells take an unique advantage as they do not cause graft versus host disease (GvHD) in allogenic stem cell transplantation (Gill *et al.*, 2009). As a result, adoptive transfer of expanded NK cells is on the way to become the new standard of care of hematological malignancies and some solid tumors (Oh *et al.*, 2019). In order to obtain therapeutic cell dose of NK cells, different expansion methods have been developed. These techniques include, but not limited to, feeder cell-based techniques (Cho *et al.*, 2009; Fujisaki *et al.*, 2009; Granzin *et al.*, 2016), plasma membranes particles or particle-based method (Oyer *et al.*, 2016), stromal support (Oyer *et al.*, 2016), and cytokine-based techniques (Decot *et al.*, 2010; Koehl *et al.*, 2005; Masuyama *et al.*, 2016; Spanholtz *et al.*, 2010).

Since lymphopenia is a well-known side effect of the traditional anticancer treatment, it is, however, not always possible to expand clinically relevant doses of NK cells from heavily treated patients (Young *et al.*, 2019). To overcome this problem, several studies have reported the feasible use of cord blood (CB) as an

alternative source of NK cells (reviewed in Zhao *et al.*, 2020). Moreover, despite its worldwide availability, cord blood-derived NK cells (CBNK cells) have been shown to have a better bone marrow homing ability when compared with peripheral blood derived NK cells (Zhao *et al.*, 2020). So far, CBNK expansion rely on the use of either artificial antigen-presenting (aAPC or feeder) cell-based techniques or cytokines-based techniques. However, in addition to its effectiveness, the presence of aAPC or feeder cells in the culture system open up an opportunity to introduce undesirable cell type into the final product (Halme *et al.*, 2006). For this reason, cytokines-based techniques are more applicable. Cytokines-based techniques employ the use of interleukin (IL)-2 either alone (Xing *et al.*, 2010) or in combination with cytokine cocktail (Spanholtz *et al.*, 2010), tacrolimus and dalteparin sodium (Tanaka *et al.*, 2012), bisphosphonate zoledronic acid (Ma *et al.*, 2018), and group A streptococcus (Mu *et al.*, 2019). Although satisfy number of expanded CBNK cells have been reported from some of these studies, no one has yet come up with known NK cells stimulatory agents: Notch signaling activator, and lipopolysaccharide (LPS). Early studies have shown that activation of Notch signaling promote NK cell differentiation from $CD34^{+}$ hematopoietic stem cells (HSCs) (Benne *et al.*, 2009; Haraguchi *et al.*, 2009). Apart from Notch signaling activator, It is demonstrated that LPS 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).

In this study, purified functional NK cells were expanded from cord blood derived mononuclear cells (CB-MNCs) using feeder-free and cell-sorting-free approaches. This protocol would pave the way to establish a sustainable supply of NK cells for clinical applications.

1.2 Research objectives

1.2.1. To promote CBNK cell proliferation by stimulating unsorted CB-MNCs with LPS or Yhhu-3792.

1.2.2 To set-up large-scale expansion protocol for CBNK cell production.

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CHAPTER II

LITERATURE REVIEW

2.1 NK cell-based immunotherapy

Growing evidences in the field of adoptive immunotherapy have shed light on the impact of Natural killer cells (NK cells) in cancer treatment. In principle, NK cell-based immunotherapy is a type of cancer treatment that use NK cells to fight against cancer. The reason was that NK cells, named by their inheritance killing ability, are cytotoxic lymphocyte endowed with array of germline-encoded surface receptors that recognize and trigger cytotoxic response to virally-infected, stressed, or cancerous cells in the absence of prior sensitization or antigen presentation (Shaver *et al.*, 2021). Role in immunosurveillance of NK cells is mediated by the net balance between signals from activating receptors (e.g., natural killer group 2 member D (NKG2D), DNAX Accessory Molecule-1 (DNAM-1), and the natural cytotoxicity receptors NKp30, NKp44, and NKp46) and inhibitory receptors (e.g., killer immunoglobulin-like receptors (KIR) and NK group 2 member A (NKG2A)) (Hu *et al.*, 2019). To date, more than one tumor recognition models of NK cells have been proposed, figure 2.1: missing-self, induced-self, and antibody-dependent cell-mediated cytotoxicity (ADCC) model (Morvan *et al.*, 2016). When the activating signals overcome inhibitory signals (a process known as “licensing”), NK cells destroy target cells through an array of killing machineries, figure 2.2. Directly, NK cells lyse target cells through the release of lytic granules containing perforin and granzymes, expression of death ligands (death receptor pathway), and activation of the antibody-dependent cell-mediated cytotoxicity (ADCC) cascade. Indirectly, NK cells mediate their killing activities by secreting inflammatory cytokines (e.g., interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α)) and chemokines (e.g., interleukin-8 (IL-8), CCL5, and XCL1) to recruit and activate the adaptive immune response (Barnes *et al.*, 2021). This important linkage between innate (NK cells) and adaptive immune responses (e.g., T cells, B cells, dendritic cells) provides a complete framework for

establishing the cancer-immunity cycle, ensuring efficient control of cancer growth (Ramírez-Labrada *et al.*, 2022).

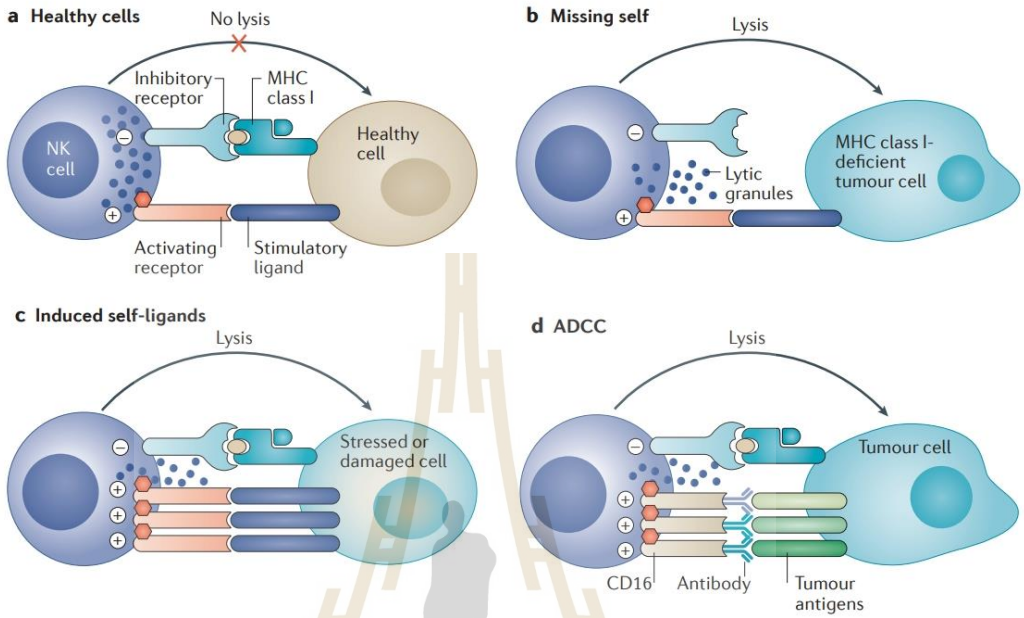

Figure 2.1 Tumor recognition models of NK cells. A) In healthy cells, prevention of self-lytic attack governs by inhibitory signals from inhibitory receptors that turn off cytotoxic responses of NK cells when recognize major histocompatibility complex (MHC) class I molecules on the surface of healthy normal cells. B) Missing self-recognition model; low or absent expression of inhibitory ligands (MHC class I molecules) on virally infected cells or transformed cells turn on NK cell cytotoxic responses. C) Induced self-recognition model; the expression of activating receptor ligands (e.g., MICA, MICB, UL16-binding protein, hemagglutinin of influenza virus (Vogler et al., 2022)) on damaged or stress cells led to the activation of NK cell cytotoxicity. D) ADCC model; the binding between FcγRIII receptor (CD16) of NK cells surface and antigen-specific antibodies initiate ADCC cascade. Reference picture from Morvan & Lanier, 2016.

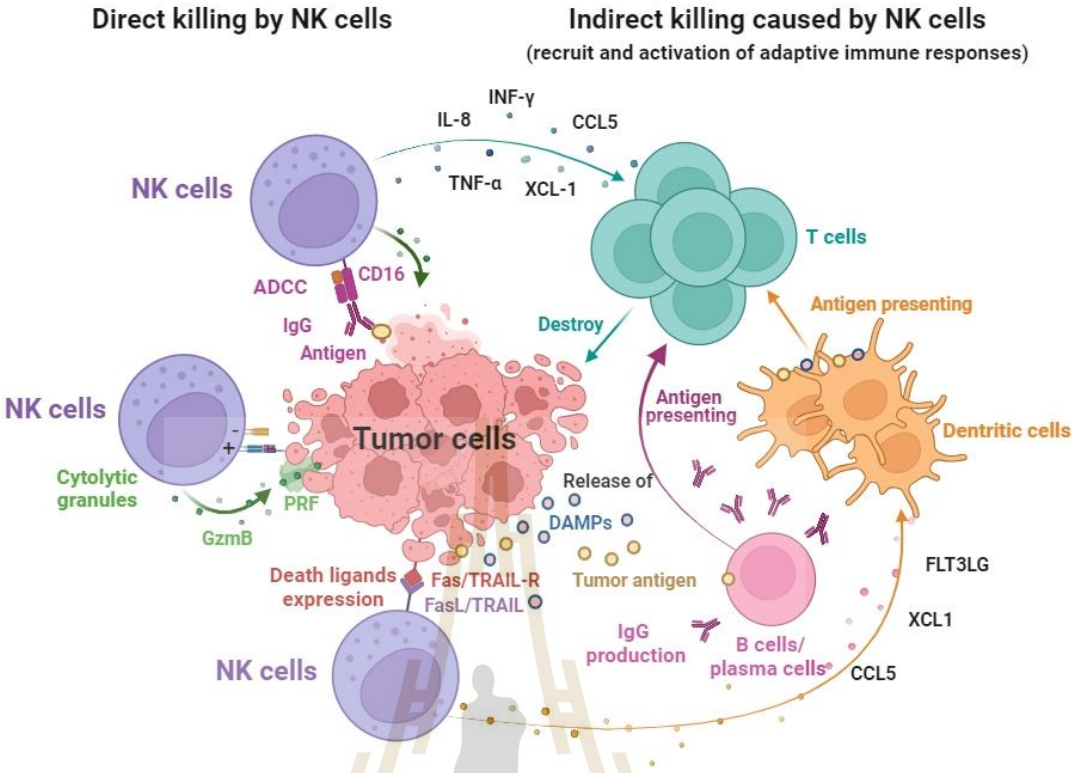

Figure 2.2 Mechanisms of NK cells cytotoxicity. After licensing, NK cells mediate cancer killing through both direct (left) and indirect (right) pathways. In the direct pathway, the elimination of target cells involves various mechanisms, such as synthesizing and releasing perforin (PRF; induces pore formation) and granzymes (GzmB; initiates apoptosis cleavage of the intracellular substrates), triggering cancer cell apoptosis through the expression of death ligands (Fas ligand; FasL and Tumor Necrosis Factor-related apoptosis-inducing ligand; TRAIL), and activating the ADCC cascade by expressing FcγRIII (CD16) to recognize target cells coated with antibodies (IgG). In the indirect pathway, NK cells recruit players from the adaptive immune system to mediate additional cancer elimination by secreting diverse types of immunomodulatory cytokines (e.g., interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF)) and chemokines (e.g., interleukin-8 (IL-8), CCL5, and XCL1). After recognizing and engulfing Danger-Associated Molecular Patterns (DAMPs) and tumor antigens, dendritic cells subsequently present processed antigen fragments to

activate T cell responses. Upon stimulation by tumor antigens, B cells contribute to cancer elimination by performing dual functions: acting as antigen-presenting cells and producing antibodies (IgG) (Prokopeva *et al.*, 2024). Modified picture from Barnes *et al.*, 2021. Created with BioRender.com.

The feasibility and safety of NK cells in cancer immunotherapy have been studied for several decades. In 1999, a pioneering study presented by Ruggeri and colleague demonstrated that donor NK cells exerted graft versus leukemia effects on recipient's target cells in acute myeloid leukemia patients underwent haploidentical hematopoietic stem cell transplantation (HSCT) (Ruggeri *et al.*, 1999). Since then, similar findings have been reported in different kinds of malignancy: acute myeloid leukemia, chronic myeloid leukemia, myelodysplastic syndrome, multiple myeloma, B cell non-Hodgkin lymphoma, neuroblastoma, renal cell carcinoma, melanoma, ovarian cancer, and breast cancer (reviewed in Shimasaki *et al.*, 2020).

2.2 Umbilical cord blood as a source of NK cells for cancer treatment

To observe a clinical benefit, sufficient numbers of NK cells with intact cytotoxic function are required. Several studies demonstrated that NK cells can be expanded from various cell sources; peripheral blood mononuclear cells (PBMCs), umbilical cord blood (CB), induced pluripotent stem cells (iPSCs), and NK cell lines (reviewed in Kundu *et al.*, 2021). Among all the options, considerable evidences suggested that CB are an attractive source of NK cells. Advantages of using CB cell source include, but not limited to, (i) CB contain substantial numbers of HSCs which possess the ability to generate all types of blood cells (Till *et al.*, 1961), (ii) aside from HSCs, CB contains a higher percentage of NK cells than peripheral blood; 15-20% versus 10-15%, respectively (Fang *et al.*, 2019), (iii) CB banking is currently available worldwide, (iv) CB units obtained from accredited cord blood bank were screened to be free form known specific infectious pathogens (Armitage, 2016), (v) fewer T cells exist along with lower risk of GvHD (Zhao *et al.*, 2020).

To exploit the therapeutic properties of umbilical cord blood-derived NK cells (CBNK cells) in cancer, completed and ongoing clinical trials have been or are being

undertaken (Table 2.1). Moreover, it was worth mentioning that the CBNK cells are currently commercially available as preserved living medicine. oNKord®, invented by Glycostem, is the first-generation of off-the-shelf cryopreserved CBNK cells product that received an orphan drug designation for AML from the United States food and drug administration (FDA) and European medicines agency (EMA) (Veluchamy, 2020). Results from phase 1 trial demonstrated that 4 out of 10 treated AML patients had a temporary disease-free period up to 60 months after infusion (Dolstra *et al.*, 2017). To further evaluate its safety and efficacy, this product is now move to phase I/IIa trial, refer to NCT04632316, in 2020.

Table 2.1 Cord blood-derived NK cells (CBNK cells) in clinical trials.

Non-genetically modified cells					
NCT number	**Cancer types**	**NK cells platform**	**Cell number**	**Phase**	**Status**
NCT01914263	ZR751, MCF7, HepG2, SMMC-7721, Hela, A375, DU145, H1299 and A549	Cytokine induced CBNK cells, cytokine induced PBNK cells	$8x10^9$ cells, single dose	I	Unknown
NCT03019640	MCL, B-cell NHL	Expanded CBNK cells	ND	II	Completed
NCT02955550	MM	Expanded CBNK cells	ND	I	Completed
NCT01729091	MM	Expanded CBNK cells	ND	II	Active, not recruiting
NCT02781467	AML	Expanded CBNK cells	ND	I	Terminated
NCT02280525	CLL	Expanded CBNK cells	1×10^7 cells/kg.	I	Completed
NCT02727803	CML, ALL, MDS, Leukemia, MM	Unexpanded CB	ND	II	Recruiting
NCT01464359	AML	T-cell depleted CB (double unit CB transplants)	ND	II	Terminated
NCT02781467	AML	Expanded CBNK cells (named PNK-007)	ND	I	Terminated
NCT04632316	AML	Expanded CBNK cells (named oNKord®)	ND	I/II	Recruiting

Table 2.1 (Cont).

Genetically modified cells					
NCT number	**Cancer types**	**NK cells platform**	**Cell number**	**Phase**	**Status**
NCT04347616	AML	Expanded CBNK cells combination with subcutaneous IL-2	1.0-3.0 x 10^9 cells	I/II	Recruiting
NCT03420963	Relapsed or refractory solid tumors	Expanded CBNK	ND	I	Recruiting
NCT05110742	T-cell malignances, MCL, CLL	CD5-IL15 CAR-CBNK cells	1 x 10^7 cells, 1 x 10^8 cells, 1 x 10^9 cells, 1 x 10^{10} cells	I/II	Not yet recruiting
NCT05092451	B cell lymphoma, MDS, AML	CAR.70/IL15-transduced CB-NK	ND	I/II	Recruiting
NCT04796675	ALL, CLL, NHL	CD19 CAR-CBNK cells	1 x 10^5 cells, 1 x 10^6 cells, 1 x 10^7 cells	I	Recruiting
NCT03056339	B cell lymphoma, ALL, CLL, NHL	CD19-CD28-zeta-2A-iCasp9-IL15- CAR-CBNK cells	ND	I/II	Completed
NCT04991870	Glioblastoma	TGF-betaR2 -/NR3C1 - CBNK cells	ND	I	Recruiting
NCT05842707	B-cell NHL	DualCAR-NK19/70	ND	I/II	Recruiting
NCT05667155	B-cell NHL	CB dualCAR-NK19/70	2×10^6 cells/kg, 4×10^6cells/kg, 8×10^6 cells/kg	I	Recruiting
NCT05472558	B-cell NHL	CB anti-CD19 CAR-NK	2×10^6 cells/kg, 3×10^6 cells/kg, 4×10^6 cells/kg	I	Recruiting
NCT06083883	Synovial cell sarcoma, myxoid/round cell liposarcoma	NY-ESO-1 TCR/IL-15 NK	ND	I/Ib	Not Yet Recruiting

Table 2.1 (Cont).

Genetically modified cells					
NCT number	**Cancer types**	**NK cells platform**	**Cell number**	**Phase**	**Status**
NCT06066424	Solid tumor (breast)	TROP2-CAR-NK	ND	I	Recruiting
NCT06066359	MM	NY-ESO-1 TCR/IL-15 NK	ND	I/II	Recruiting
NCT03579927	B-cell NHL	CAR CD19-CD28-zeta-2A-iCasp9-IL15-transduced CBNK	ND	I/II	Withdrawn
NCT05703854	RCC, mesothelioma, osteosarcoma	(CAR).70/interleukin (IL)15-transduced CBNK	ND	I/II	Recruiting
NCT05922930	Ovarian cancer, mesonephric-like adenocarcinoma, pancreatic cancer	TROP2- CAR-NK	ND	I/II	Recruiting

Data from ClinicalTrial.gov, accessed on 8/2/2024, search term: cord blood-derived NK cells, cancer, and oNKord. Abbreviation: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CAR, chimeric antigen receptor; CLL, chronic lymphocytic leukemia; HCC, hepatocellular carcinoma; MCL, mantle cell lymphoma; MDS, myelodysplastic syndromes; MM, multiple myeloma; ND, not determined; NHL, non-hodgkin's lymphoma; RCC, renal cell carcinoma.

2.3 CBNK cells expansion platform

Different platforms have been proposed to generate clinically relevant CBNK cell dose for adoptive cell therapy. Either artificial antigen-presenting cells (aAPCs or feeder cells)-based techniques or cytokines-based techniques have been reported.

2.3.1 Feeder cell-based techniques

In 2013, Shah and colleague have shown that cord blood derived mononuclear cells (CB-MNCs) can be expanded and can give rise to a mean of 1,848-fold and 2,389-fold expansion of NK cells (with > 95% NK cell purity) from fresh and cryopreserved CB, respectively (Shah *et al.*, 2013). In this study, the expansion took

place in GP500 bioreactor in the presenting of 100 IU/ml IL2 and K562-based aAPCs expressing membrane bound (mb) IL-21 “Clone9.mbIL21” at 2:1 aAPCs:cell ratio. Immunomagnetically CD3-depletion were performed on day 7 and the expansion were last for a total of 14 days. In 2015, Vasu *et al.* were demonstrated that a median of 6,092 x 10^6 $CD3^-CD56^+$ NK cells (range between 165–20,947 x 10^6 cells) can be obtained from only 1 ml of post-thaw CB unit after 35 days of expansion (Vasu *et al.*, 2015). In this study, the authors developed a selective access to cryopreserved samples device and employed the use of irradiated Epstein-Barr virus-transformed human B-cell lymphoblastoid cell lines, at 20:1 aAPCs:cell ratio, to stimulate and to expand 1 x 10^6 CD3-depleted total nuclear cells obtained from thawed CB in the medium containing 500 IU/ml IL2. A further study to evaluate the potential use of aAPCs to expand CBNK cells was published in by Ayello *et al.*, 2017 . In their study, they used irradiated genetically modified K562 cells expressing the NK-stimulatory molecules 4-1BB ligand and interleukin 15 (also known as K562-mbIL15-41BBL cell line) to co-cultured with CB-MNCs, at 1:1 aAPCs:cell ratio, in RPMI-1640 medium containing 10 IU/ml IL2. After 7 days of expansion, they demonstrated that the percentage of $CD3^-CD56^+$ NK cells significantly increased from 3.9% (at day 0) to 71.7±3.9%, 42.6±5.9%, and 9.7±2.4% in culture conditions contained K562-mbIL15-41BBL cells, wide-type K562 cells, and media alone, respectively. In addition, the same study also showed that the *in vitro* cytotoxic activity against B-cell non-Hodgkin lymphoma (B-NHL) of K562-mbIL15-41BBL-expanded CBNK cells was significantly higher than wide-type K562-expanded CBNK cells (40±3.1 vs. 18±2.1, respectively). Although a remarkable potential to expand CBNK cells has been reported from all of these studies, the use of tumor cell derived aAPCs/feeder cells rise a safety concern on final product purity (Halme *et al.*, 2006).

2.3.2 Cytokines-based techniques

Cytokines-based approaches to generate large numbers of functionally competent CBNK cells have been described since 2010. Xing and team showed that an average 92-fold (range between 39–112-fold) expansion of CBNK cells could be obtained from $CD3^-CD56^+$ sorted CB-MNC cells after 14 days of culture in RPMI-1640 medium containing 10% human serum and 500 IU/ml IL2 (Xing *et al.*, 2010). In the same year, Spanholtz *et al*. published a two-step cytokine-based protocol that could

efficiently generated more than 10,000 x 10^6 CBNK cells from freshly selected $CD34^+$ CB-MNC cells (Spanholtz *et al.*, 2010). In this study, $CD34^+$ CB-MNC cells were first immunomagnetically sorted and expanded for 14 days in medium containing 10% human serum and cytokine cocktails: 10 pg/ml GM-CSF, 250 pg/ml G-CSF, 50 pg/ml LIF, 200 pg/ml MIP-Iα, 50 pg/ml IL6, 27 ng/ml SCF, 25 ng/ml FLT-3L, 25 ng/ml TPO, 20 ng/ml IL15, and 25 ng/ml IL7. These cells were then differentiated and further expanded for another 21 days in NK cell differentiation medium containing 10% human serum and cytokines: 10 pg/ml GM-CSF, 250 pg/ml G-CSF, 50 pg/ml LIF, 200 pg/ml MIP-Iα, 50 pg/ml IL6, 20 ng/ml IL7, 22 ng/ml SCF, 1,000 IU/ml IL2, and 20 ng/ml IL15. Next, in 2012, Tanaka *et al.* reported that proliferation of CBNK cells was achieved by supplementation of 10 ng/ml IL15, 5 ng/ml IL2, 10 – 1,000 ng/ml anti-CD3 monoclonal antibody (clone OKT3), 0.02 - 0.1 ng/ml tacrolimus and 5 – 10 IU/ml dalteparin sodium to the medium. (Tanaka *et al.*, 2012). In this study, the authors highlighting the beneficial effects of tacrolimus and dalteparin sodium as a T cell anti-proliferation and NK cell stimulation agent, respectively. After 3 weeks of expansion, about 40 x 10^6 CBNK cells with the maximum of 72.8% NK purity were obtained. In addition, Ma *et al*. found that a combination of high concentration of IL2 (2,000 IU/ml) and 5 μM zoledronate could possibly induce a greater expansion of CBNK cells (average 1,286-fold) from CB-MNCs (Ma *et al.*, 2018). Without cell sorting, this method yields up to 80.46% NK cell purity after 21 days of culture. Last but not least, Mu *et al.* employed the use of immunopotentiator extracted from group A streptococcus to reinforce CBNK cell proliferation (Mu *et al.*, 2019). In this study, CB-MNCs were first stimulated for 3 days in AIM-V medium supplemented with 2,000 IU/ml IL2, 0.01 KE/ml group A streptococcus, and 5 μM zoledronate. Then, the activated cells were expanded in the same medium containing only 2,000 IU/ml IL2 for 21 days. In the end of the process, this method could produce up to an average of 15,900 x 10^6 CBNK cells with more than 90% NK cell purity.

2.4 Approches to improve CBNK cell expansion

2.4.1 Notch signaling activator

During NK cell development, NK cell follow a carefully orchestrated journey starting from $CD34^+$ HSCs to common lymphoid progenitors and subsequently

to NK cell progenitors (Bi *et al.*, 2020). The pleiotropic functions of NOTCH signaling pathway have long been reported to plays an essential role in various physiological processes include cell proliferation, cell fate determination, and cell death (Wang, 2011). Activation of Notch signaling combined with NK associated cytokine milieu have been reported to have a significant role in *in vivo* NK cell development (Bachanova *et al.*, 2009; Rolink *et al.*, 2006). Under feeder-free expansion system and in the presence of Notch ligand delta 4, IL7, SCF, and FLT-3L, human cord blood $CD34^+$ cells could effectively differentiate into functional NK cells (Haraguchi *et al.*, 2009). A significant therapeutic value of small molecules that modulate Notch pathway has been reviewed by Sail *et al.*, 2012 . Azelaic acid, a nine-carbon dicarboxylic acid NOTCH signaling activator, have been found to promote the proliferation of PBNK cells (Dongdong *et al.*, 2019). Under a concentration of 10 μM/ml azelaic acid plus 100 IU/ml IL2, $CD3^-CD56^+$ sorted PBNK cells could proliferate nearly 1.5-fold after 24 hrs. of stimulation. Beyond PBNK cell proliferation, the aforementioned study highlighted that an additional advantage of using small molecules is a conformational stability of the substance. Among a larger collection of a newly synthesized small molecule drug, Yhhu-3792 is a 2-phenylamino-quinazoline-based compound (figure 2.3) which potentially activates the NOTCH signaling pathway. In mouse neural stem cell model, Yhhu-3792 treatment led to an increase in the diameter and number of neurospheres compared with the untreated group (Lu *et al.*, 2018). Based on the positive effect on promoting the self-renewal of neural stem cells, Yhhu-3792 is currently of great interest for its potential additional clinical applications.

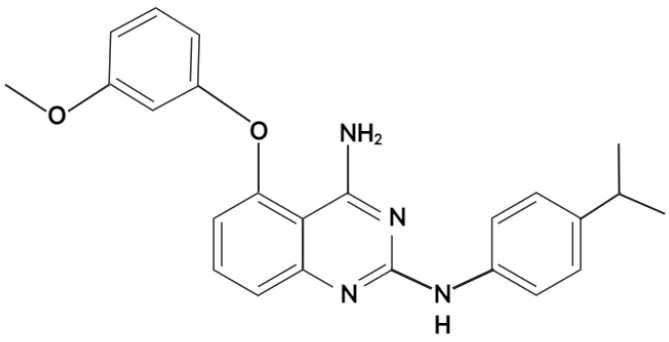

Figure 2.3 Structure of Yhhu-3792. Created with BioRender.com.

2.4.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major structural component of an outer membrane of most gram-negative bacteria envelope that owes an ability to stimulate the immune response (Bertani *et al.*, 2018). Typically, LPS consist of 3 structural domains named Lipid A, core oligosaccharide, and O antigen (figure 2.4). A line of evidence suggests that LPS is a potent activator of macrophages, T cells, and NK cells (Conti *et al.*, 1991; Mattern *et al.*, 1994; McAleer *et al.*, 2008). Study underpinning the role of LPS on PBNK cell proliferation has been published. In 2000, Goodier and Londei demonstrated that the percentage of $CD3^{-}CD56^{+}$ PBNK cells increased from 10.5±3.5% (range 3.8-16.7%) to 20.9±11.3% (range 9.1-48.4%) after stimulated with 1 μg/ml LPS for 9 days, before and after stimulation respectively (Goodier *et al.*, 2000). Later, it was discovered that LPS can directly promote IFN-γ production of PBNK cells (Kanevskiy *et al.*, 2013). Moreover, in the field of vaccine industry, synthetic LPS have long been pursued as a adjuvant of human vaccine (Zariri *et al.*, 2015).

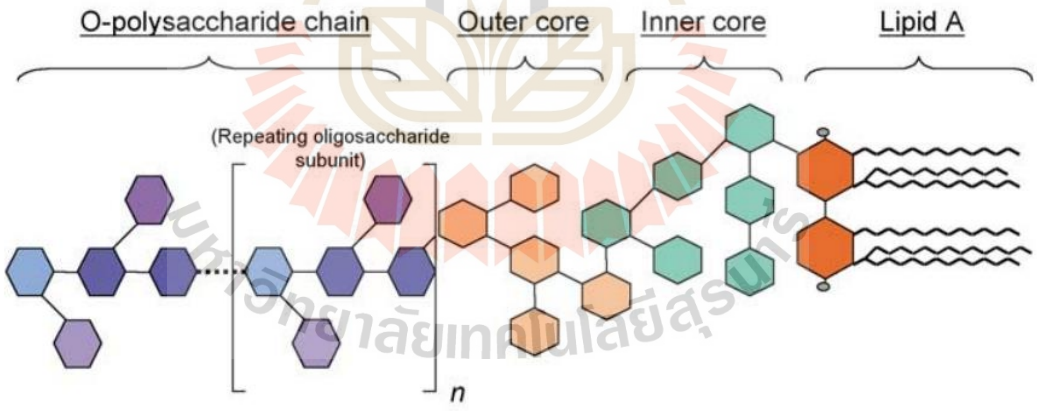

Figure 2.4 Structure of lipopolysaccharide. The classic LPS molecule consists of three main structural components: lipid A, core oligosaccharide, and O antigen. The immunogenic activity of LPS varies among different bacterial species, depending on the variation in lipid A and O antigen structure (Lin *et al.*, 2020). Reference picture from Erridge *et al.*, 2002.

2.5 Challenges in generating CBNK cells for adoptive cell transfer

2.5.1 Anti-cancer activity of CBNK cells

Although adoptive cell transfer (ACT) of *ex vivo* expanded CBNK cells is an attractive alternative source to PBNK cells, an important consideration when using CBNK cells is the functional activity of the cells. It has been reported that variations in the phenotype and function of NK cells exist among those derived or isolated from different sources: PBNK cells, CBNK cells (expanded from $CD34^+$ or $CD56^+$ sorted populations), and iPSC-derived cells, figure 2.5 (Goldenson *et al.*, 2020).

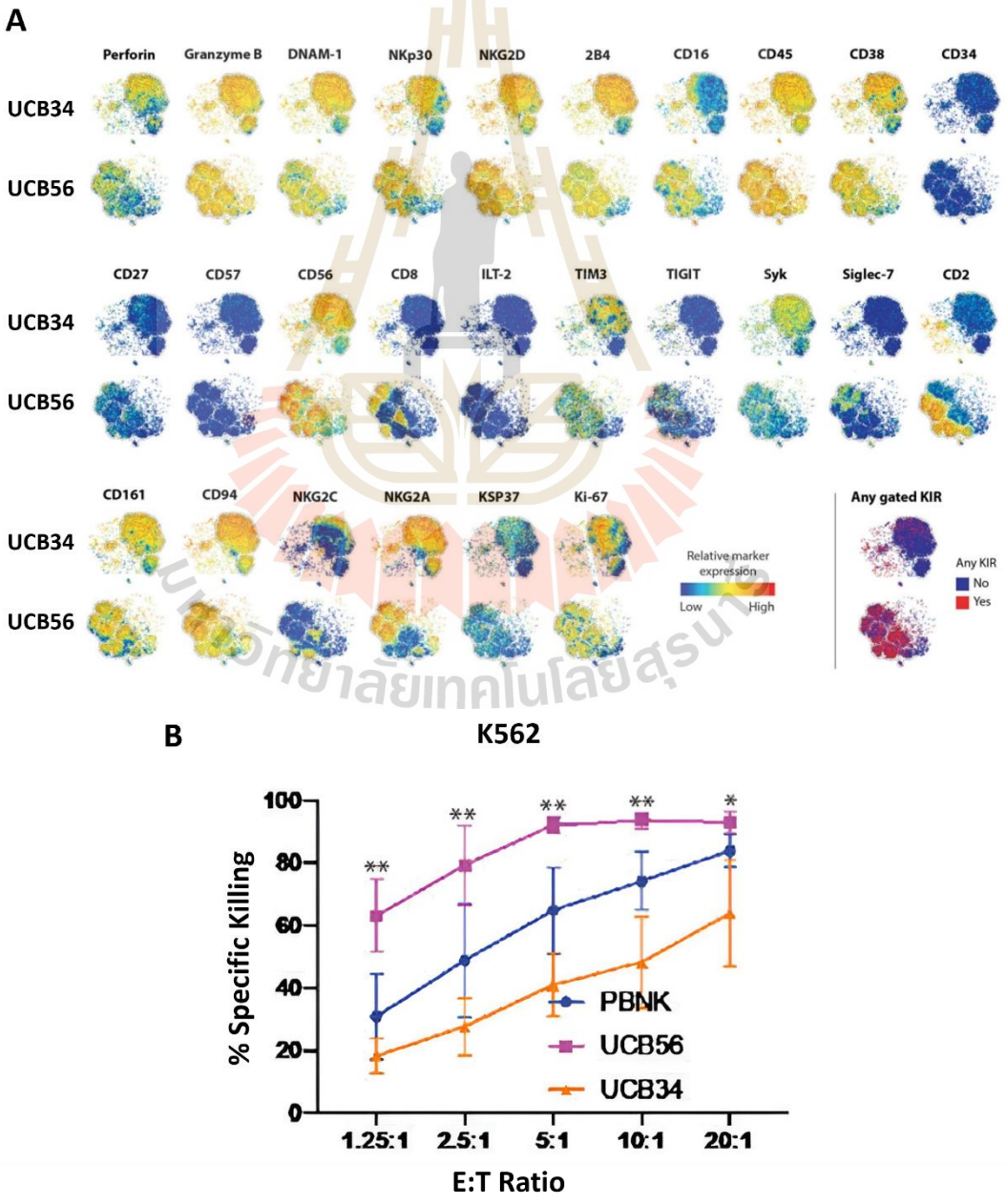

Figure 2.5 Distinct characteristics of NK cells. A) Representative t-distributed Stochastic Neighbor Embedding (t-SNE) plot from mass cytometry analysis showing the expression of NK cell surface markers in NK cells expanded from $CD34^+$ (UCB34) and $CD56^+$ (UCB56) populations obtained from the umbilical cord blood of the same donor. B) Analysis of NK cell killing activity using caspase-3/7 green apoptosis assay. UCB34-NK, UCB56-NK, and PBNK cells show differences in their cytolytic ability against K562 cells. Reference from Goldenson *et al*., 2020.

As shown in figure 2.5A, results from mass cytometry analysis reveal significant differences in the expression of CD16 and CD2 between NK cells expanded from CD34+ (UCB34) and CD56+ (UCB56) sorted populations, irrespective of their shared UCB origin (Goldenson *et al.*, 2020). Moreover, the same study also shows that UCB56 derived NK cells (UCB56-NK) displayed a more potent cytotoxicity compared to PBNK cells and UCB34 derived NK cells (UCB34-NK) (figure 2.5B). Inaddition, anti-cancer activity of the expanded CBNK cells varies between studies. In 2012, Luevano *et al*. demonstrated that expanded CBNK cells failed to lyse K562 cells and produced less IFN-γ than PBNK cells. (Luevano *et al.*, 2012). This lower activity could be explained by their immature phenotype, marked by the downregulation of key activating receptors (e.g. NKG2D, CD16, NKp30, NKp44 and NKp46) coupled with the upregulation of inhibitory receptors (e.g. NKG2A) (Shokouhifar *et al.*, 2021). Conversely, efficient expansion procedures for the production of functionally competent CBNK cells have been reported. For example, Kang *et al*. developed a 21-day feeder cell-based cultivation process, yielding a substantial number of highly cytotoxic CBNK cells that have the ability to kill a wide variety of tumor cell models (figure 2.6) (Kang *et al.*, 2013).

Taken together, all the studies described above indicate unique characteristics (both phenotype and function) of the expanded CBNK cells obtained from different approaches. Therefore, it would be indispensable to assess the anti-cancer activity of the expanded CBNK cells before transferring this technology into a clinical setting.

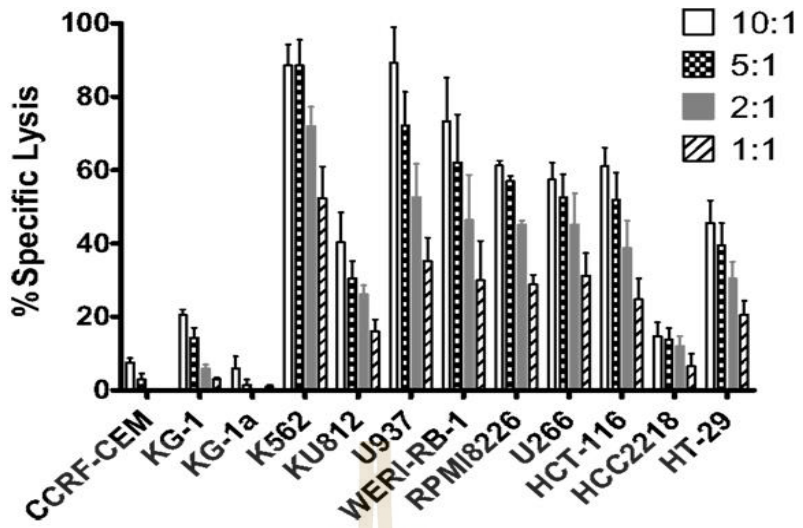

Figure 2.6 Anti-cancer activity of the expanded CBNK cells. Results of the lactate dehydrogenase assay demonstrated that expanded CBNK cells destroy target cancer in a dose-dependent manner. Reference picture from Kang *et al*., 2013.

2.5.2 Manufacturing practice of CBNK cells

To fulfill the fast-paced development of NK cell-based immunotherapy, common challenges in the cell therapy industry include scaling up production and implementing quality controls and release criteria. Due to the nature of adoptive cell therapy (ACT) as a living treatment, its success depends on both the appropriate dosage and the integrity of the transferred cells (figure 2.7). In a clinical trial setting, the number of transferred NK cells ranges from 5×10^6 to 1×10^8 cells per kilogram of body weight (Heipertz et al., 2021). Given this range, a single dose of NK cell treatment for an adult weighing 70 kilograms is between 35×10^7 to 7×10^9 cells. Therefore, the commercial-scale production of NK cell products requires a large-scale expansion system that can handle liters, hundreds, or thousands of liters of culture working volume per batch. To reach this goal, bioreactors are now the favored choice in NK cellmanufacturing, facilitating efficient and scalable production processes.

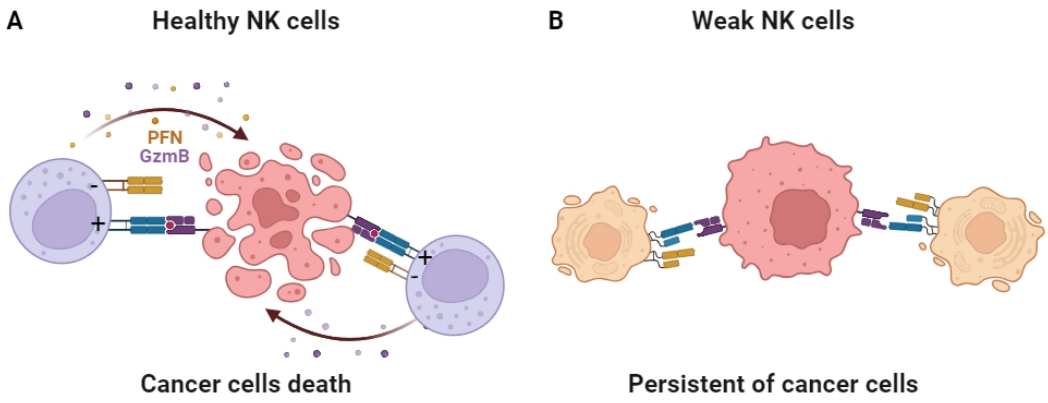

Figure 2.7 Efficiency of the living treatment. Healthy NK cells mediate effective cancer killing (A), while weak or nearly dying NK cells exert minimal to no response to cancer cells (B). Created with BioRender.com.

In addition, to comply with Good Manufacturing Practice (GMP) standards, it is advisable to choose a single-use, closed, and disposable cell production kit with an automatic system (Garcia-Aponte *et al.*, 2021). This helps avoid most technical complexities, such as handling multiple T-flasks or bags simultaneously, and minimizes the risk of errors and contamination. To date, automatic and semi-automatic manufacturing systems that have been reported to support NK cell production include CliniMACS Prodigy® from Miltenyi Biotec (Albinger *et al.*, 2024; Granzin *et al.*, 2015), Zellwerk's Z®RP platform from Zellwerk GmbH (Bröker *et al.*, 2019), and Xuri Cell Expansion System W25 from Cytiva (Veluchamy, 2020). The existence of batch-to-batch variation in NK cell products stems from various factors, including variations in the phenotypes of initially seeded cells, differences in cytokine and medium due to lot-to-lot inconsistency, and variations in process conditions such as dissolved oxygen, carbon dioxide, and pH. Therefore, it would be wiser to employ process analytical technology (e.g., raman spectroscopy, sequential injection capillary electrophoresis, optical biosensors, electrical impedance, etc.) to monitor and control the cell expansion process (Garcia-Aponte *et al.*, 2021). In line with this, real-time in-line monitoring, label-free, and contact-free techniques are more favorable to consider.

Like many types of medicinal products, quality controls and release criteria should be taken into great consideration. Since regulations and regulatory agencies can vary from country to country, the cell therapy industry should understand and follow the regulatory framework and legislation for cell therapy products in each country. The two biggest regions leading in this field are the European Union (EU) and the United States (US). In EU, cell therapy products were classified as "advanced therapy medicinal products" (ATMPs), while in US, NK cell products were classified as Cell and Gene Therapy Products (CGT) (Salazar-Fontana, 2022). The classification and definition of ATMPs and CGT products also differ. For instance, ATMPs are divided into somatic cell therapy medicinal products (SCTMP), tissue-engineered products (TEP), gene therapy medicinal products (GTMP), and combined ATMPs (cATMPs), while CGT products are simply categorized into two groups: gene therapy and cellular therapy products. EMA/CHMP/BWP/271475/2006 rev.1 and FDA-2008-D-0520 are specific guidance for cell therapy Industry on potency testing of ATMPs and CGT products, respectively (Iglesias-Lopez *et al.*, 2019). Overall, cardinal requirements of these guidelines include the identity, quality, purity, strength, and stability of the product.

Lastly, but most importantly, storing and distributing off-the-shelf NK cell products may inevitably require a cryopreservation process. In the cell therapy industry, cryopreservation is a useful technique that facilitates long-term storage of the living cells. Technically, cryopreservation provides extra time for many vital activities, including quality tests, donor and recipient matching (if needed), long-distance product delivery, and multiple treatments of the same batch. Unfortunately, the common problems of cryopreserved NK cells include cell loss caused by multiple washing step of freezing/thawing process, cell damage due to the toxicity of cryoprotectant agents (CPAs) (Lamers-Kok *et al.*, 2022), and a significant drop in cytotoxicity. Recently, results from the standard 2-dimensional (D) chromium release assay on fresh and cryopreserved (frozen-thawed) expanded NK cells demonstrated that the cytotoxicity of cryopreserved NK cells significantly declined when compared to fresh NK cells, figure 2.8A (Mark *et al.*, 2020). To investigate the underlying mechanisms of this decrease, the same study employs a 3-D collagen gel matrix coupled with time-lapse imaging and reveals that the loss in antitumor activity of the cryopreserved NK cells is caused by a reduction in motile NK cells, figure 2.8B. To

solve this problem, a variety of approaches have been reported, Table 2.2 (modified from Saultz *et al.*, 2023).

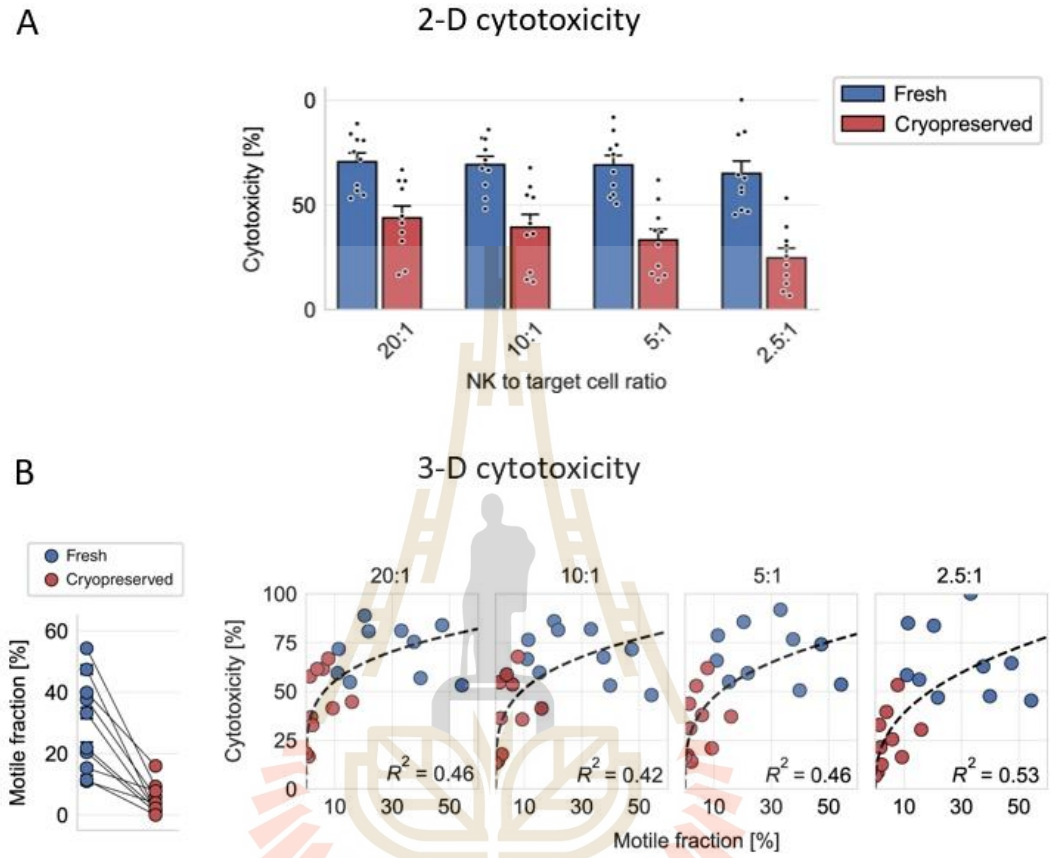

Figure 2.8 Effect of cryopreservation on NK cell cytotoxicity. A) In 2-D conditions, decreased cytotoxicity was observed on cryopreserved NK cells but not on fresh NK cells. B) Results of pairwise experiments on 3-D cell motility (left) and 3-D cell cytotoxicity (right) reveal that the fraction of motile NK cells decreased after cryopreservation, and this reduction is significantly related to lower cytotoxicity. For all tested NK-to-target cell ratios. Reference picture from Mark et al., 2020.

Table 2.2 Different approaches for a better outcome of NK cells post-thaw recovery.

No.	Cell concentration/cryomedia/container	Freezing/storage condition	Thawing /post-thaw recovery	Outcome	Reference
1.	**Cell density:** 1-5×10^6 cells/mL. **Cryomedia 1:** human AB serum + 10% DMSO **Cryomedia 2:** DMEM + 10% human AB serum + 10% DMSO **Container:** ND	**Freezing:** ND **Storage:** LN_2	**Thawing:** 1:10 dilution in media **Post-thaw:** ND	**Cell viability:** does not affected **Cell cytotoxicity:** does not affected	(Domogala *et al.*, 2016)
2	**Cell density:** 1×10^8 cells/mL. **Cryomedia:** RPMI 1640 + 20% albumin + 25% dextran-40 + 5% DMSO **Container:** ND	**Freezing:** ND **Storage:** LN_2	**Thawing:** 37°C water bath, slow diluted with RPMI1640 + 10% FBS **Post-thaw:** no recovery step (analyzed immediately)	**Cell viability:** ruduce from 95.9%±0.5% to 92.2%±0.8% **Cell cytotoxicity:** NS between fresh and cryopreserved NK cells.	(Min *et al.*, 2018)
3	**Cell density:** 1-2.5×10^7 cells/mL. **Cryomedia:** 50% RPMI1640 + 40% FBS, 10% DMSO **Container:** Cryotube vial	**Freezing:** by Mr. Frosty™ freezing container (-80°C, overnight) **Storage:** LN_2	**Thawing:** 37 °C water bath, dropwise 5-fold dilution in RPMI 1640 + 10% FBS + 1% antibiotic **Post-thaw:** recovery in RPMI 1640 + 10% FBS + 1% antibiotic +100 IU/mL IL2 (16 h, overnight thaw)	**Cell viability:** does not affected and can be recovered **Cell cytotoxicity:** thawed and rested (16 h) NK cells showed no decrease in cytotoxicity compared to fresh NK cells.	Oyer *et al.*, 2022

Abbreviation: CRF, Controlled Rate Freezer; DMSO, Dimethyl sulfoxide; FBS, fetal bovine serum; ND, not determined; NS, not significant different; RPMI 1640, Roswell Park Memorial Institute 1640 medium

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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.

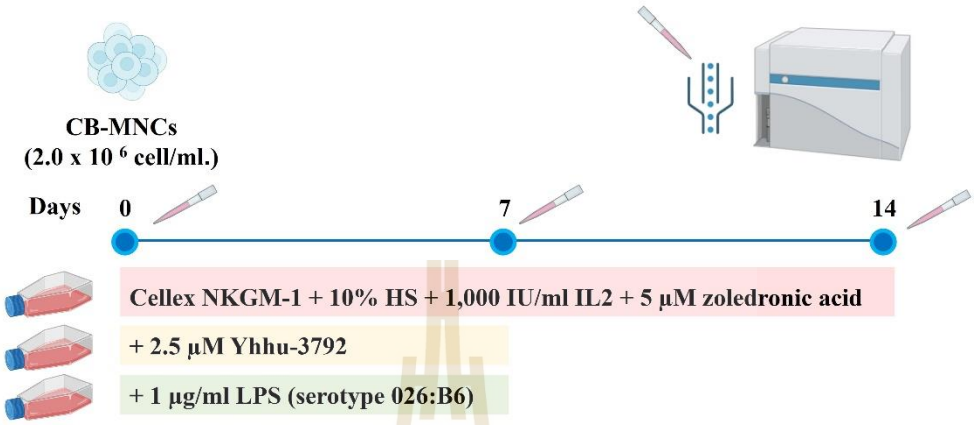

Figure 3.1 The schematic diagram of the experiment used to stimulate the expansion of CBNK cells.

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).

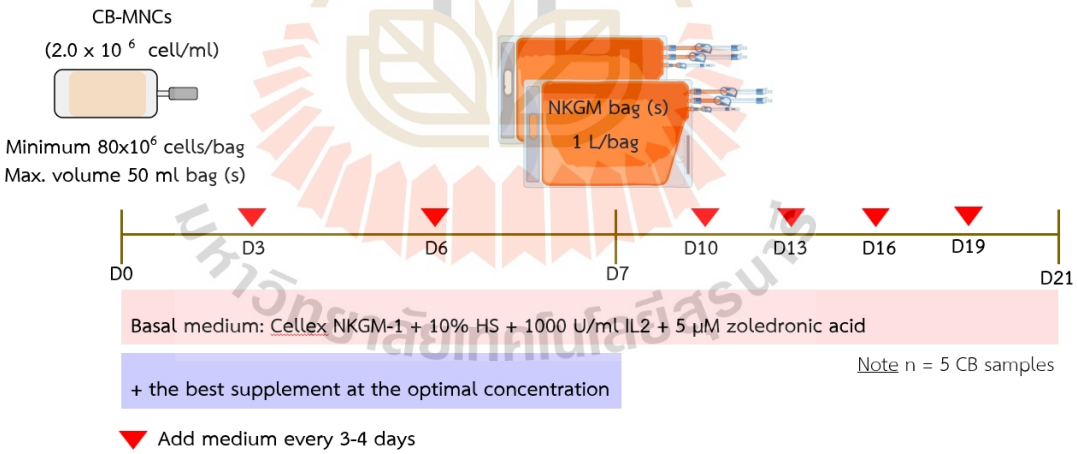

Figure 3.2 The schematic diagram of the upscaling expansion experiment.

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).

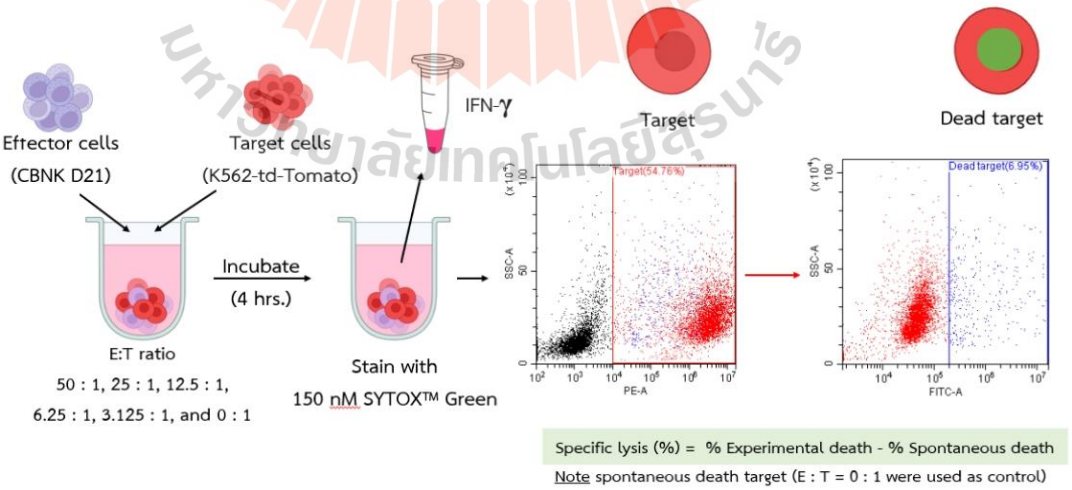

Figure 3.3 The schematic diagram of the flow cytometry-based NK cytotoxicity assay.

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.

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

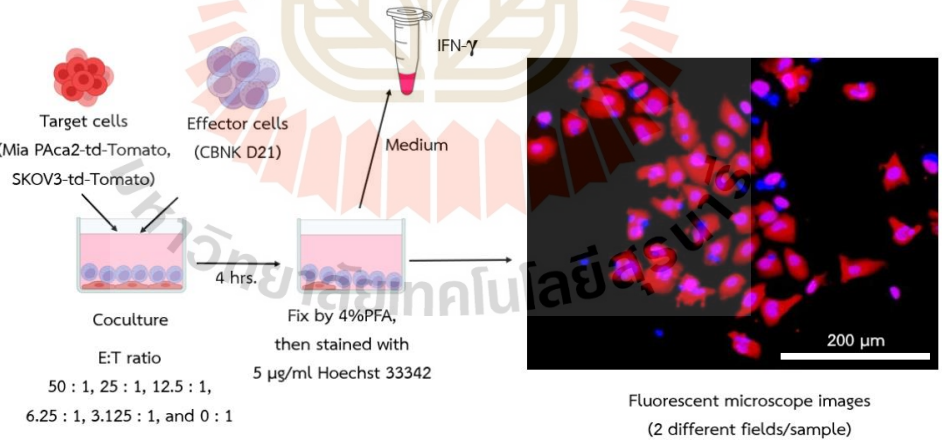

Figure 3.4 The schematic diagram of the microscopic-based NK cytotoxicity assay.

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).

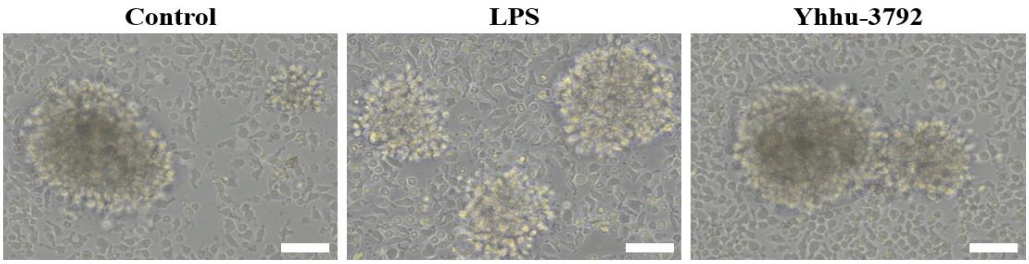

Figure 3.5 Morphology of the expanded cells at day 14. The bar represents 50 µm, corresponding to 20x magnification.

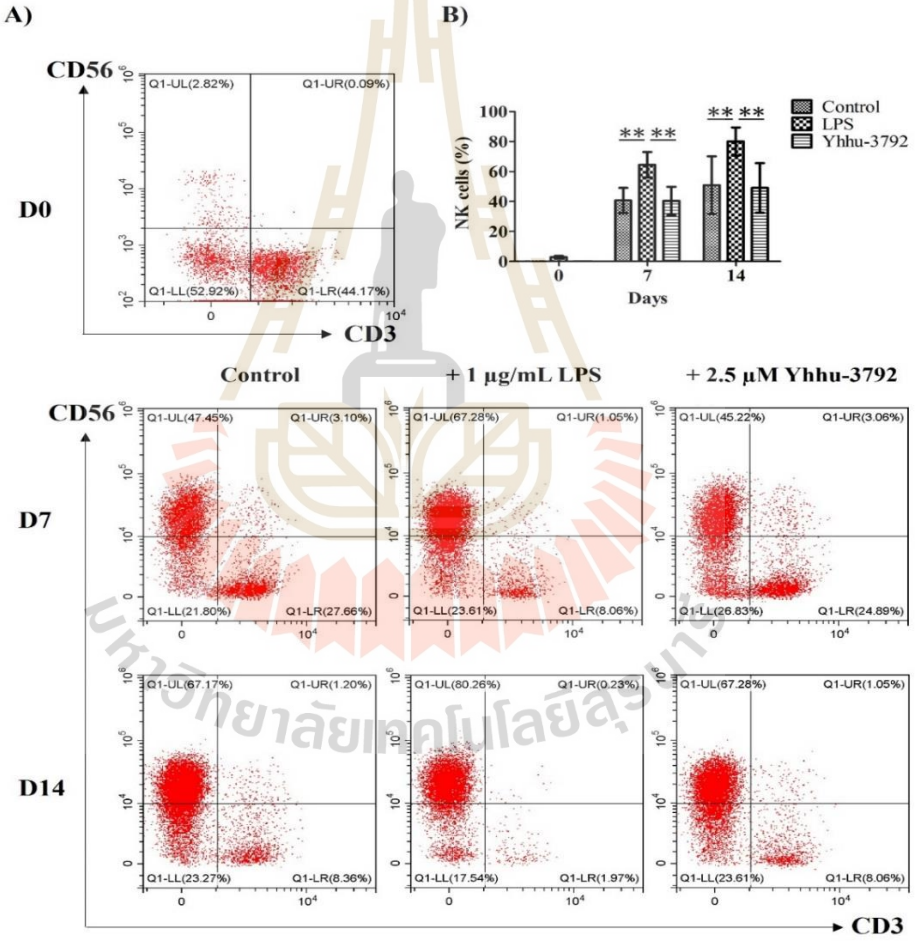

Figure 3.6 The effect of LPS and YHHU on proportion of CBNK cells. A) Representative flow cytometry dot plots of $CD56^{+}CD3^{-}$ NK cells on day 0, day 7, and day 14 under LPS and YHHU stimulation. B) The percentage of $CD56^{+}CD3^{-}$ NK cells growth under tested conditions. Data are expressed as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

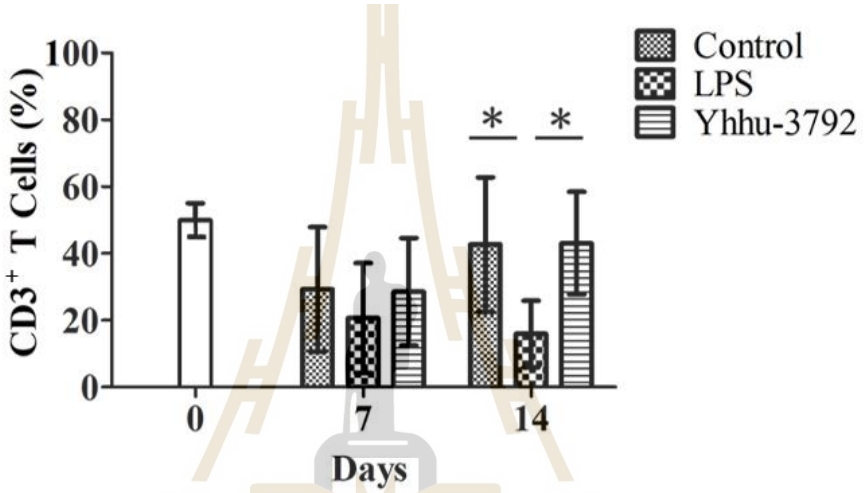

Figure 3.7 The percentage of $CD3^{+}$ T cells over the 14-day expansion. Data are expressed as the mean ± SEM

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.

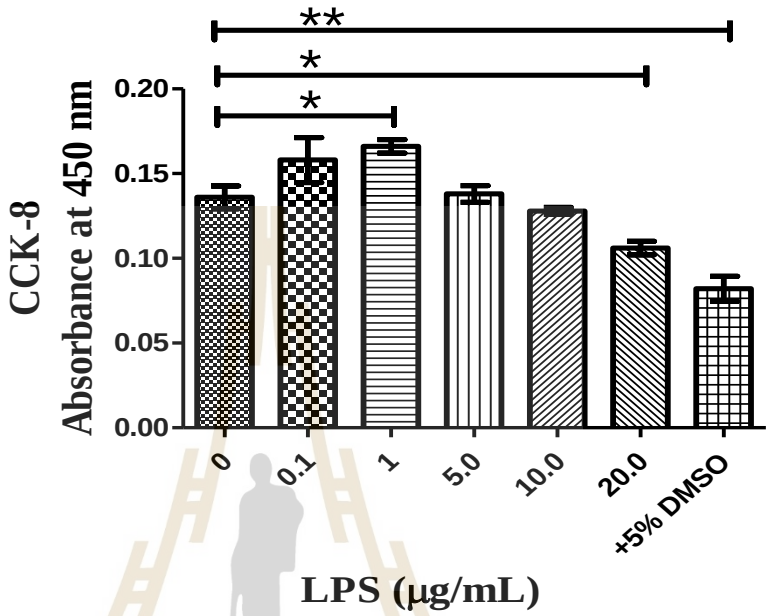

Figure 3.8 The effect of LPS concentration on CCK-8 response. Data are expressed as the mean ± SEM of OD values.

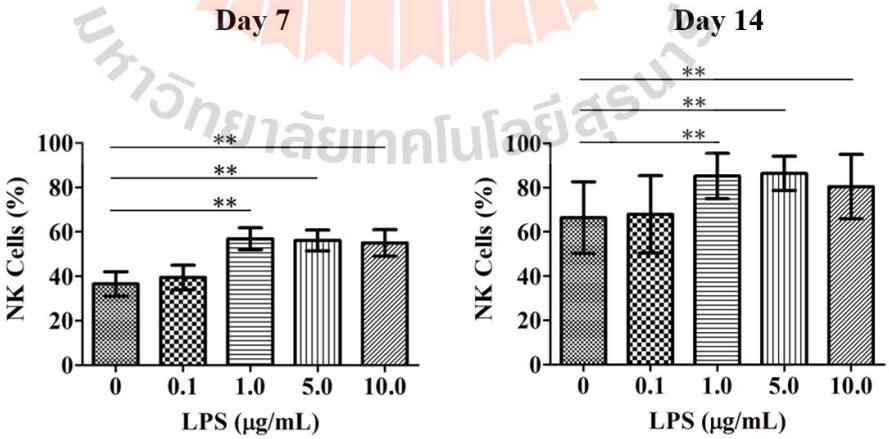

Figure 3.9 The percentage of NK cells in different LPS concentrations. Data are expressed as the mean ± SEM (n = 5).

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).

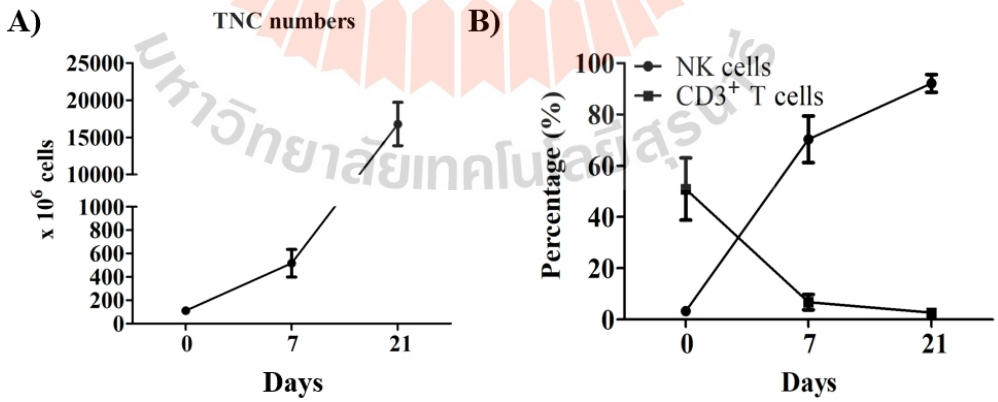

Figure 3.10 Upscaling expansion of NK cells using the LPS stimulation approach. A) Average yield of TNCs expansion and (B) the proportion of NK and T cells on day 0, day 7, and day 21. Data are expressed as the mean ± SEM (n = 5).

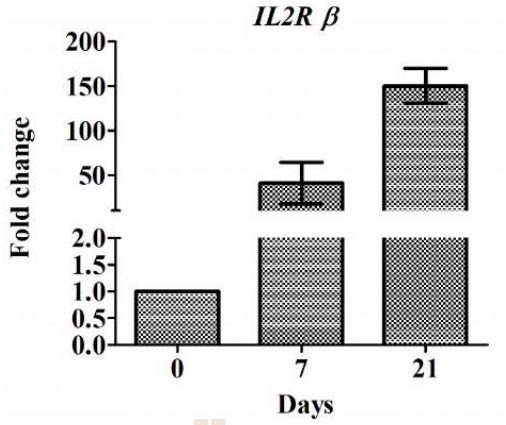

Figure 3.11 The gene expression levels of *IL2Rβ*. The expression values were normalized to *GAPDH*. Data are expressed as the mean ± SEM.

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.

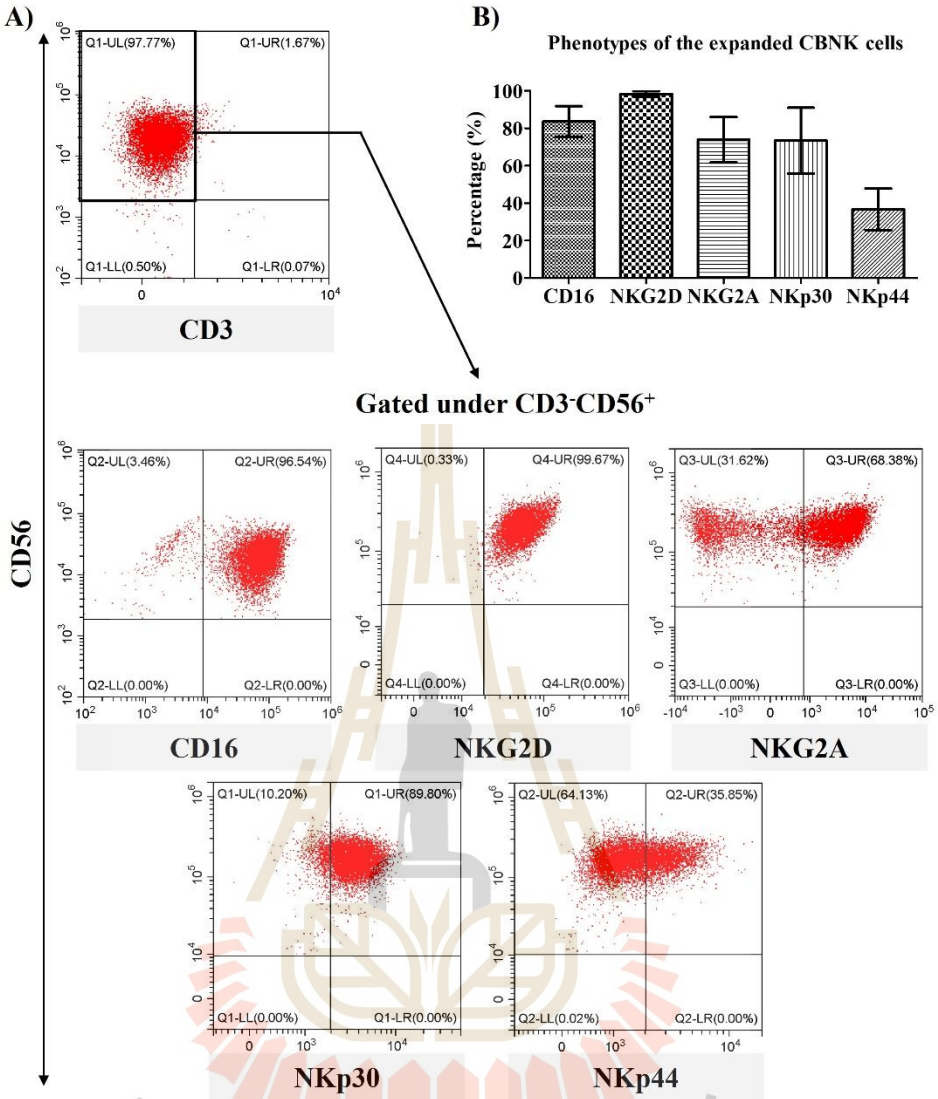

Figure 3.12 Immunophenotype profile of the expanded cells. A) Representative flow cytometry dot plots of the expanded cells (day 21). B) The percentage of NK cell-associated markers on the expanded cells (day 21). Data are expressed as the mean ± SEM (n = 5).

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).

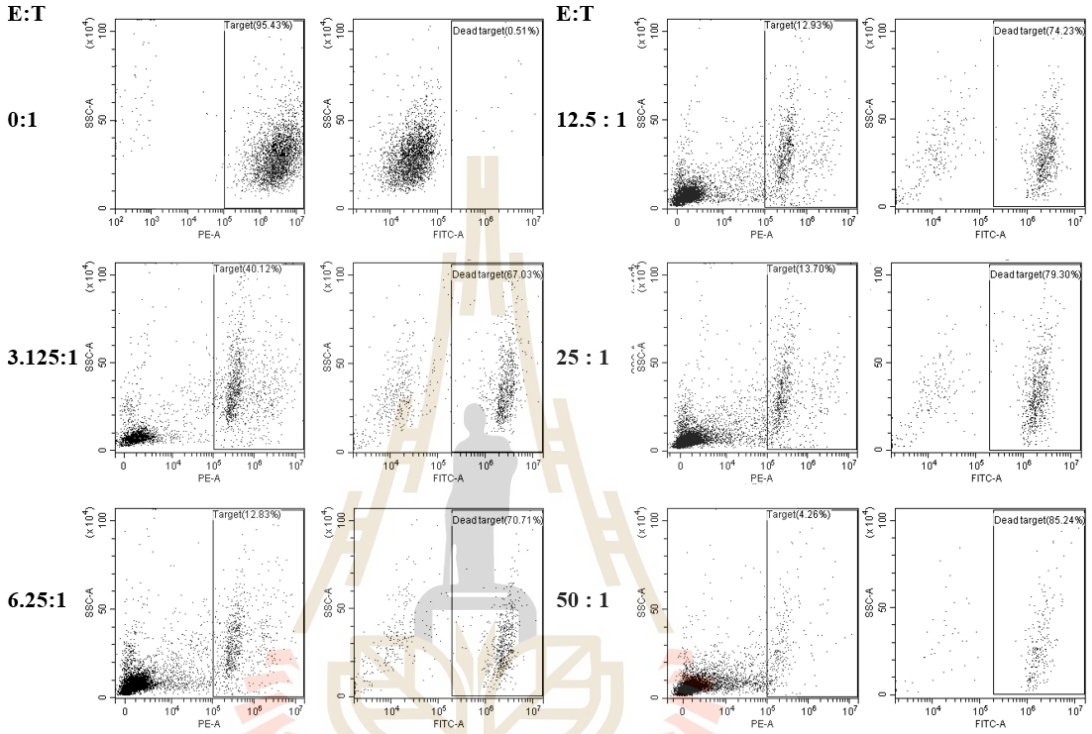

Figure 3.13 Cytotoxicity of the expanded CBNK cells against K562 tdTomato-tagged cells. Representative dot plot showing the initial gate (left; PE-A/SSC-A plot) and subsequent target cell plot (right; FITC-A/SSC-A plot). Dead targets were FITC-positive cells gated from the PE-positive population.

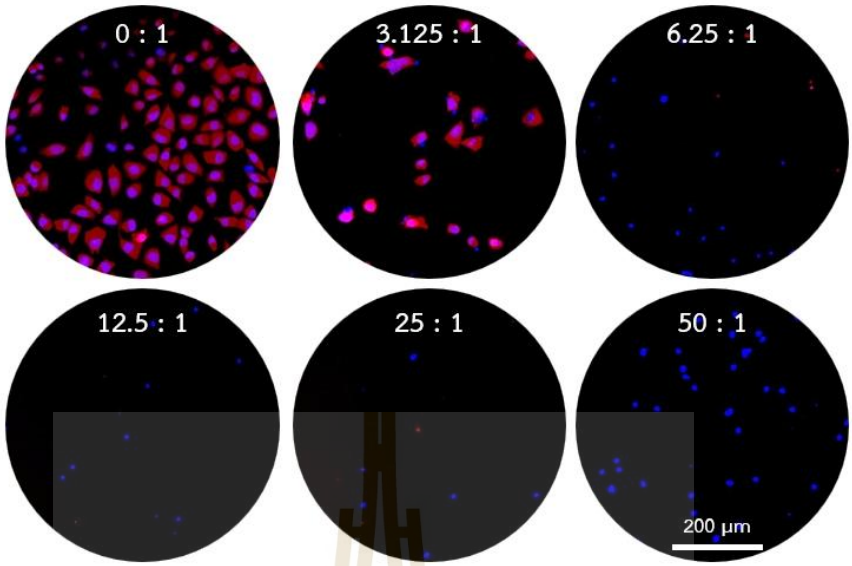

Figure 3.14 Cytotoxicity of the expanded CBNK cells against MIA PaCa-2 tdTomato-tagged cells. Representative fluorescence microscopic image of MIA PaCa-2 cells (red) after 4 hours of co-incubation with expanded CBNK cells (n = 5). The bar represents 200 µm, corresponding to 10x magnification.

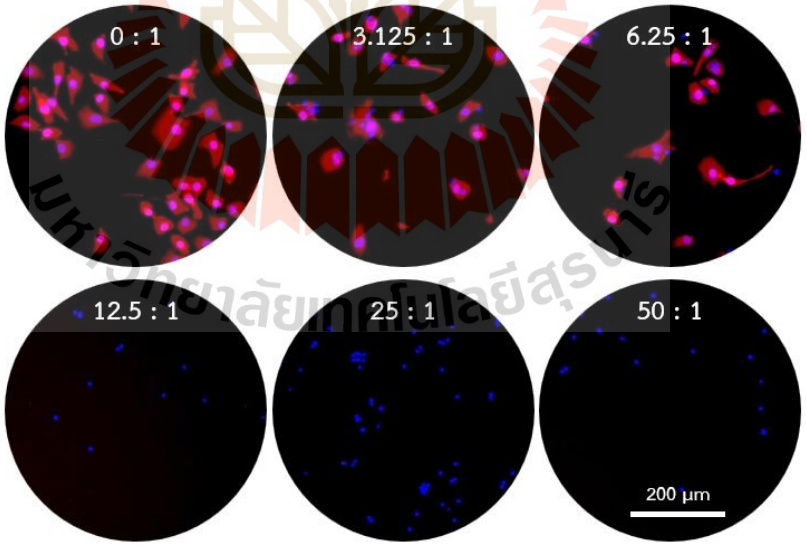

Figure 3.15 Cytotoxicity of the expanded CBNK cells against SKOV3 tdTomato-tagged cells. Representative fluorescence microscopic image of SKOV3 cells (red) after 4 hours of co-incubation with expanded CBNK cells (n = 5). The bar represents 200 µm, corresponding to 10x magnification.

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.

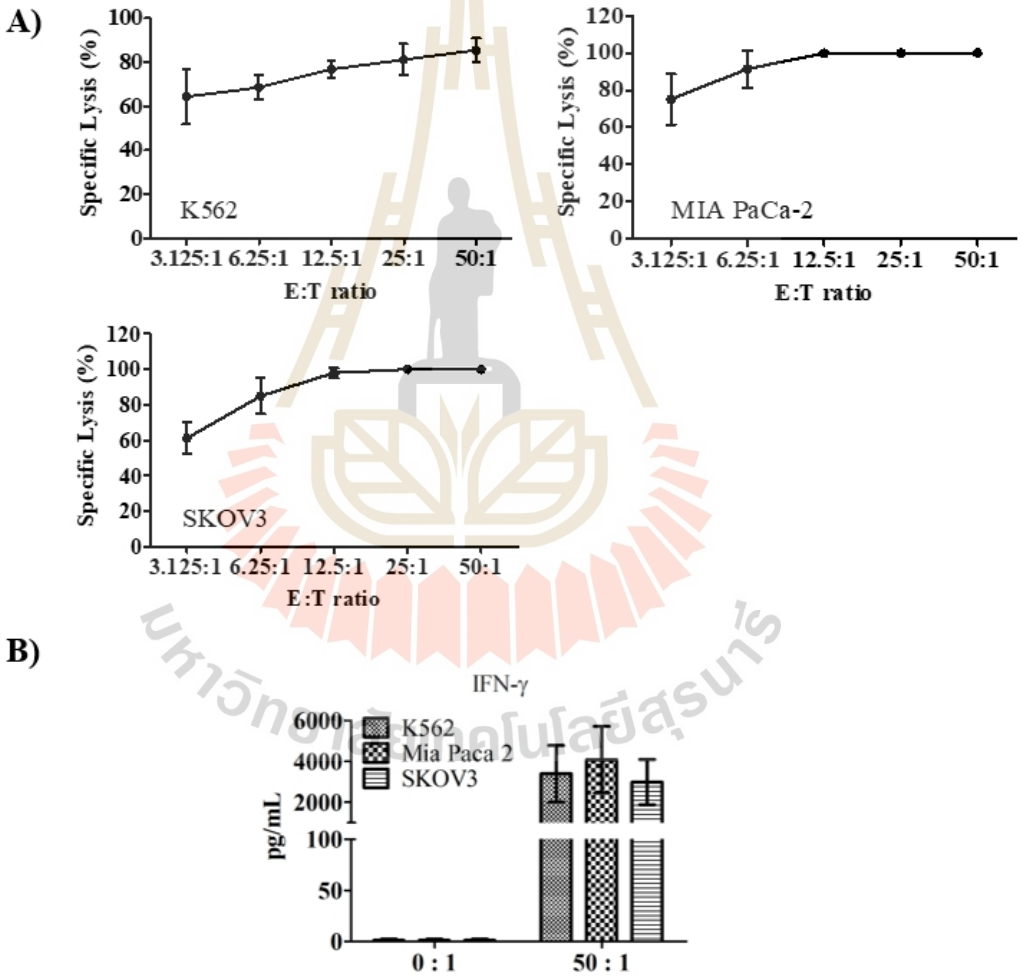

Figure 3.16 Anti-cancer activities of expanded CBNK cells. A) NK cell cytotoxicity tests against K562, MIA PaCa-2, and SKOV3 cells. B) IFN-γ secretion in co-culture media during cytotoxicity testing. Data are expressed as the mean ± SEM (n = 5).

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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CHAPTER IV
OVERALL CONCLUSION

To develop a sustainable supply of NK cells for ACT, the driving of NK cell lineage commitment of CB-MNCs during the first week of expansion is a promising strategy that greatly takes advantage of its developmental potential. Under a feeder-free and cell-sorting-free approach, induction of CBNK cell proliferation was achieved by supplementation with 1 μg/mL LPS. Further upscaling expansion reveals that CBNK cells generated by this technique are composed of both quantification and qualification. This finding serves as the initial step in the development of off-the-shelf CBNK cells as a medicinal product for cancer treatment.

APPENDIX

Table A1 Antibodies used for flow cytometry analysis.

Antibodies	Companies	Catalogue No.
FITC anti-human CD45	BioLegend	304006
PE anti-human CD34	BioLegend	343506
PerCP/Cyanine5.5 anti-human CD3	BioLegend	300430
APC/Cyanine7 anti-human CD56 (neural cell adhesion molecule; NCAM)	BioLegend	362512
PE anti-human CD16	BioLegend	302008
PE anti-human CD314 (NKG2D)	BioLegend	320806
APC anti-human CD159a (NKG2A)	BioLegend	375108
PE/Dazzle 594 anti-human CD337 (NKp30)	BioLegend	325232
Alexa Fluor 647 anti-human CD336 (NKp44)	BioLegend	325112
PE anti-human CD107a (Lysosome-Associated Membrane Protein 1; LAMP-1)	BioLegend	328608
FITC Mouse IgG1, Isotype Ctrl	BioLegend	400138
PE Mouse IgG1, Isotype Ctrl	BioLegend	400114
PerCP/Cyanine5.5 mouse IgG1, isotype Ctrl	BioLegend	400150
PE/Dazzle 594 mouse IgG1, isotype Ctrl	BioLegend	400176
APC mouse IgG1, isotype Ctrl	BioLegend	400120
Alexa Fluor 647 mouse IgG1, isotype Ctrl	BioLegend	400136
APC/Cyanine7 mouse IgG1, isotype Ctrl	BioLegend	400128

BIOGRAPHY

Hataiwan Kunkanjanawan was born on 18th October 1983, in Bangkok, Thailand. She earned a Doctor of Veterinary Medicine degree from the Faculty of Veterinary Medicine, Kasetsart University, in 2008. After that, she pursued a Master's degree in 2010 at the School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, and graduated in 2013. Following her studies, she joined Medeze Research and Development Co. Ltd. as a researcher. Since then, she has been dedicatedly working in the field of cancer immunotherapy and stem cells, aiming to advance medical treatments and improve patient outcomes. To pursue her career path as a dedicated and passionate researcher, she commenced her Ph.D. program at the School of Biotechnology, Institute of Agricultural Technology, SUT in 2020. Her research topic is “Expansion of purified natural killer cells from human umbilical cord blood”. Her Ph.D. study received support from the Medeze Research and Development fund and One Research One Graduate fellowships from SUT under the guidance of Assoc. Prof. Dr. Rangsun Parnpai. The research findings from Chapter 3 of her thesis will be published as a research article.