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STUDY ON THE EXPANSION AND ACTIVATION OF NATURAL KILLER (NK) CELLS FROM UMBILICAL CORD BLOOD FOR SUPPORTING CANCER THERAPY

Major: Biotechnology

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SUMMARY OF

APPLIED BIOLOGY DOCTORAL THESIS

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Reviewer 3: ….

The thesis will be defended before the Doctoral Thesis Evaluation Council of the Academy, held at the Academy of Science and Technology – Vietnam Academy of Science and Technology at … hours, on …..… 2025

The thesis is available for reference at:

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INTRODUCTION

1. The urgency of the thesis

Cancer remains a major global health challenge, prompting extensive research to identify effective treatment strategies. Recently, immune cell–based therapies have gained significant attention, employing various cells such as natural killer (NK) cells, cytotoxic T lymphocytes (CTLs), cytokine-induced killer (CIK) cells, and dendritic cells (DCs) [1–3]. Among these, NK cells are particularly effective in recognizing and destroying cancer cells through multiple pathways.

In autologous transplantation, NK cells isolated from the cancer patient may have impaired functionality or fail to recognize target cells when reintroduced into the body. In contrast, allogeneic NK cell transplantation offers several notable advantages, such as a lower risk of graft-versus-host disease (GVHD), higher functional activity of NK cells derived from healthy donors, and enhanced therapeutic efficacy due to HLA–KIR mismatches between donor and recipient.

Among immune cell sources, umbilical cord blood provides a promising supply of immune cells for allogeneic NK cell therapy in cancer treatment, offering benefits such as ease of collection and isolation. In Vietnam, however, no institution has yet conducted studies on NK cells derived from cord blood, despite its potential as an effective adjunctive cancer therapy.

Based on these practical considerations, we conducted this study: "***Research on expansion and activation of natural killer cells (NK) from umbilical cord blood for supporting cancer treatment***"

2. Scientific and practical significance

*** Scientific significance**

- The study characterizes the populations of cord blood cells, including cell counts, the proportion of hematopoietic stem cells (HSCs), and the composition of immune cell subsets in cord blood, as well as the correlations among these parameters.
- The study further describes the characteristics and dynamics of NK cell populations during in vitro expansion, as well as the properties of NK cell clusters following culture and activation.

*** Practical significance**

- The study provides a strategy for the safe and stable in vitro expansion of NK cells from cord blood, yielding cells with high functional activity and potent antitumor capacity, with potential applications in clinical cancer therapy.
- The study also offers insights into the proportions of stem cells and immune cell subsets in cord blood, along with analyses of their interrelated factors. This serves as a foundation for neonatal immune profiling in disease prediction and diagnosis, as well as a basis for future research on other cord blood–derived immune cell therapies.

3. Research objectives

Based on the aforementioned practical considerations, this study was conducted with the following objectives:

- To analyze the characteristics and proportions of immune cell populations and hematopoietic stem cells in the cord blood of Vietnamese newborns, aiming to clarify the role of cord blood as a genuine source of stem cells and potential immune cells for therapeutic applications.
- To investigate the in vitro expansion and activation of NK cells from cord blood with high functional activity, meeting quality criteria for potential application in clinical cell therapy in the future.

4. Novel contributions of the thesis

- This study analyzed the characteristics of key immune cell populations in cord blood and identified differences in the proportions of these populations in Vietnamese newborns compared to reported data from populations in other countries.
- This research represents the first successful establishment in Vietnam of a protocol for the culture, activation, and expansion of NK cells from cord blood with high functional activity and antitumor capacity. The quality of the expanded NK cell product meets several critical criteria set by the U.S. Food and Drug Administration (FDA) for characterizing biological products at the early experimental stage, including identity, sterility, purity, safety, and therapeutic potential.

CHAPTER 1. OVERVIEW

1.1. Current cancer situation

1.1.1. Cancer situation in the world

Cancer is the second leading cause of death worldwide, accounting for an estimated 9.6 million deaths out of more than 18 million new cases in 2022. According to statistics, nearly 19 million new cancer cases were reported globally in 2022. Meanwhile, annual cancer-related deaths account for more than 50% of new cases. It is projected that by 2050, there will be over 35 million new cancer cases and more than 18.5 million cancer-related deaths worldwide each year.

1.1.2. Cancer situation in Vietnam

According to data from the International Agency for Research on Cancer (IARC) in 2022, the five most commonly diagnosed cancers in Vietnam were breast cancer (13.6%), liver cancer (13.6%), lung cancer (13.5%), colorectal cancer (9.3%), and stomach cancer (9%),

followed by other types of cancer. In Vietnam, the mortality rate among cancer patients is over 60%, which is considerably higher than the global average of approximately 50%.

1.2. Overview of Immunotherapy in Cancer Treatment

Edward Jenner, who successfully developed the first smallpox vaccine in 1796, is considered the pioneer who laid the foundation of immunology for the development of this field. The medical and scientific communities have since recognized immunotherapy as a promising approach for cancer patients, particularly those with metastatic disease or poor responses to conventional treatments [4, 5].

1.3. Current research on immune cell therapy

1.3.1. Global research status of immune cell therapy

Globally, immunotherapy has become a major focus for clinicians and researchers, not only due to its therapeutic efficacy but also because of rapid technological advancements in the field. Numerous studies worldwide have reported the safety and effectiveness of therapies such as dendritic cell (DC) therapy, CAR-T, CAR-NK, and NK cell therapies in clinical settings. Patients receiving immune cell therapy—either as a monotherapy or in combination with other treatments—have shown reductions in tumor size, improved quality of life, and extended survival…

1.3.2. Research and application of immune cell therapy in Vietnam

In Vietnam, research and application of immune cell therapy for cancer treatment, although initiated in 2011, remain at an early stage. Most publications focus on activation methods and the evaluation of antitumor efficacy of various immune cells such as dendritic cells (DCs), cytokine-induced killer (CIK) cells, and gamma delta (γδ) T cells in vitro, in mouse models, and in a limited number of clinical trials

assessing safety and efficacy—for example, autologous NK, cytotoxic T lymphocyte (CTL), and γδ T cell therapies derived from adult peripheral blood.

1.4. Overview of Natural killer (NK) cell

1.4.1. Functions of NK cells

Natural killer (NK) cells are a subset of innate lymphoid cells (ILCs) that play a critical role in innate immune responses. First identified in 1973, the term "Natural Killer" reflects their inherent ability to eliminate infected or abnormal cells without the need for prior sensitization or antigen recognition. The main functions of NK cells include immune regulation and cytotoxic activity.

1.4.2. Characteristics of Natural Killer (NK) Cells

In cord blood, NK cells account for approximately 30% of total lymphocytes, which is markedly higher than their proportion in peripheral blood, around 10% [6, 80]. Cord blood NK cells also exhibit the $CD3^{-}/CD56^{+}$ phenotype and can be divided into two subsets, CD56^dim and CD56^bright. However, the proportion of $CD16^{-}$CD56^bright cells is higher in cord blood than in peripheral blood [6, 80]. Cord blood NK cells are generally more immature and possess greater proliferative capacity compared to peripheral blood NK cells. Upon cytokine stimulation, the cytotoxic capabilities of NK cells from both sources are comparable [83, 84].

1.4.3. Surface Receptors of NK Cells

1.4.3.1. Role of Receptors

During interactions between NK cells and target cells, the outcome of NK cell activity is determined by the integration of signals from activating and inhibitory receptors on NK cells and their corresponding ligands on target cells [68]. NK cell receptors can be

classified into two main groups based on their molecular structure: (1) C-type lectin-like receptors, including NKG2 and CD94, and (2) immunoglobulin-like receptors, including natural cytotoxicity receptors (NCRs), CD16, ILT-2, and killer-cell immunoglobulin-like receptors (KIRs).

Immunoglobulin-like KIRs regulate NK cell cytotoxicity by interacting with MHC-I molecules [89]. Activating KIRs have short cytoplasmic tails and do not signal directly, whereas inhibitory KIRs possess long cytoplasmic tails containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which transmit inhibitory signals directly [90].

1.4.5. Sources and Activation Methods of NK Cells

NK cells can be obtained from peripheral blood mononuclear cells (PBMNCs), umbilical cord blood (UCB), bone marrow (BM), stem cells such as human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs), as well as commercial NK cell lines like NK-92 or NK-92MI. Each of these sources presents specific advantages and limitations regarding isolation, activation, and expansion to generate cell products that meet clinical standards [104].

1.4.6. Allogeneic NK Cell Transplantation in Cancer Therapy

To date, numerous phase I and II clinical trials have been conducted to evaluate the safety and efficacy of umbilical cord blood–derived NK cells in combination with chemotherapy or monoclonal antibodies for cancer treatment. However, a major limitation of this approach is the restricted volume of cord blood collected, which limits the number of NK cells available for transplantation. Therefore, strategies to activate cord blood–derived NK cells and enhance their cytotoxic activity require further investigation.

CHAPTER 2. SUBJECTS AND RESEARCH METHODS

2.1. Study Site, Duration, and Subjects

- The study was conducted from July 2017 to July 2021 at Vinmec Times City International General Hospital, funded by Vingroup and approved by the Ethics Committee for Biomedical Research of Vinmec International General Hospital Joint Stock Company (Approval No. 1507/2017/CN/HĐĐĐ VMEC, dated June 15, 2017).
- A total of 59 umbilical cord blood samples and 9 peripheral blood samples were selected for the study.

2.2. Research Methods

2.2.1. Sample Collection

2.2.2. Isolation of Cord Blood Mononuclear Cells Using the AXP System

2.2.3. Isolation of Cord Blood Mononuclear Cells Using Ficoll

2.2.4. Characterization of Immune Cell Populations Using Flow Cytometry

2.2.5. NK Cell Expansion

2.2.6. Expansion and Maintenance of K562 Cell Line

2.2.7. Expansion of KHYG Cell Line

2.2.8. NK Cell Activity Assay

2.2.9. Karyotyping Analysis

2.2.10. Endotoxin Quantification Using Endosafe-PTS System

2.2.11. Mycoplasma Detection Using MycoAlert Kit

2.2.12. Cell Viability Assessment Using Trypan Blue Staining

2.2.13. Nucleated Cell Counting Using Turk's Solution

2.2.14. Cryopreservation of Cells

2.2.15. Determination of Mononuclear Cell Recovery Efficiency

2.2.16. Statistical Data Analysis

Data on maternal, fetal, and cord blood characteristics were analyzed using descriptive statistics. Differences in mean values were assessed by Student's t-test or the Mann–Whitney U test, with $p < 0.05$ considered statistically significant. Pearson's linear regression analysis was used to evaluate correlations among biological factors. Figures and graphs were generated using GraphPad Prism software.

CHAPTER 3. RESEARCH RESULTS

3.1. Collection of Umbilical Cord Blood

A total of 59 eligible mothers volunteered to donate cord blood samples. Among these, 48 samples were used for mononuclear cell (MNC) isolation, and 12 samples were utilized for NK cell expansion studies.

3.1.2. Characteristics of Cord Blood

The average volume of collected cord blood samples was 94.49 ± 30.35 mL, excluding anticoagulant.

Table 3.7. Total Nucleated Cells (TNC) in Cord Blood

Total Nucleated Cells (TNC) (x 10^8 cells)	**(N=48)**
Mean (SD)	11.49 (3.97)
Median [Min; Max]	10.69 [5.10; 19.82]

Table 3.8. Total Mononuclear Cells (MNC) in Cord Blood

Total Mononuclear Cells (MNC) (x 10^8 cells)	**(N=48)**
Mean (SD)	4.34 (1.66)
Median [Min; Max]	4.44 [1.64; 8.42]

3.1.3. Number of Hematopoietic Stem Cells in Cord Blood (HSCs)

Table 3.10. Total HSCs in Collected Cord Blood Units

(N=48)	**Number of HSC (x10^6 cells)**
Mean (SD)	3.64 (2.00)
Median [Min; Max]	3.5 [0.81; 9.79]

Table 3.11. Average Number of HSCs by Gender

Gender	**Female**	**Male**	**p value**
HSC *(x10^6 cells)*	2.97 (1.51)	4.12 (2.23)	0.03

Table 3.12. Average Number of HSCs by Collected Blood Volume

Cord Blood Volume	**< 100 ml**	**> 100 ml**	**p value**
HSC *(x10^6 cells)*	2.83 (1.65)	5.26 (1.74)	**1.12 x 10^{-5}**

This study did not find the significant differences in the HSC number between groups of gestational age below 38th weeks and those above 38th weeks, whereas HSC number were lower in male infants compared to female infants (p=0.03<0.05).

3.1.4. Umbilical Cord Blood Mononuclear Cell Separation

Table 3.16. Results of MNC Isolation

	Ficoll	**AXP**
N (sample)	10	38
Sample volume (ml)		
Mean (SD)	60.27 (5.39)	103.49 (27.44)
Median [Min; Max]	58.3 [52; 68]	94.75 [68.3; 176]
MNC Pre (x10^8)		
Mean (SD)	3.21 (1.59)	3.6 (1.52)
Median [Min; Max]	2.39 [1.64; 7.21]	4.64 [1.65; 8.42]
MNC pos (x10^8)		
Mean (SD)	2.33 (1.21)	3.44 (1.43)

Median [Min; Max]	1.78 [1.34; 5.34]	4.27 [1.55; 7.78]
MNC recovery (%)		
Mean (SD)	75 (5)	96 (5)
Median [Min; Max]	75 [65; 82]	98 [79; 100]
P value	p<0.05	

3.2. Peripheral blood sample

3.2.1. Patient characteristics

A total of nine patients were enrolled in the study, including three patients with gastric cancer, three with hepatocellular carcinoma, and three with lung cancer. The mean age of the study population was 66 ± 8.8 years, with the youngest patient aged 53 years and the oldest aged 79 years.

3.2.2. Peripheral blood samples collected

*Table 3.19. Correlation between initial cultured cell counts, patient age, and cancer type (*p<0,05)*

Number of cell at day 0	**Total (x10^6)**	**% NK**	**Number of NK (x10^6)**
	(Mean± SD)	(Mean± SD)	(Mean± SD)
Medium	38.5±20.2	16.7±8.4	4.5±3.4
Aged	*p*=0.9888	*p*=0.0205*	*p*=0.2251
≤ 65	44.7±18.3	7.1±4.1	2.3±0.5
> 65	37.0 ±21.1	19.1±7.5	5.1±3.6
Cancer type	*p*= 0.588	*p*=0.089	*p*=0.002*
Gastric	34.0±16.0	9.3±3.3	2.3±0.6
Liver	39.7±26.1	22.0±5.0	5.4±2.8
Lung	45.2±19.3	21.0±11.5	7.3±5.7

3.3. Characteristics of umbilical cord blood cell populations

3.3.1. Proportion of immune cell types (T-B-NK) in umbilical cord blood

Table 3. 20. B-cell proportion by birth weight and gestational age

(N=59)	**CD3-/CD19+ (%)**		
Mean (SD)	9.52 (3.28)		
Median [Min; Max]	9.2 [3.5; 18.6]		
Birth weight (kg)	***<=3kg***	***>3kg***	***P value***
N (%)	17 (28.8%)	42 (71.2%)	
Mean (SD)	8.83 (2.82)	9.80 (3.48)	>0.05
Gestational age	***<=38 weeks***	***>38 weeks***	***P value***
N (%)	18 (30.5%)	41 (69.5%)	
Mean (SD)	8.34 (2.36)	10.04 (3.55)	**<0.05**

Table 3.21. $CD3^+/CD19^+$ cell proportion by birth weight and gestational age

(N=59)	**CD3+/CD19+ (%)**		
Mean (SD)	8.59 (5.77)		
Median [Min; Max]	8.30 [0.1; 20.4]		
Birth weight (kg)	***<=3kg***	***>3kg***	***P value***
N (%)	17 (28.8%)	42 (71.2%)	
Mean (SD)	5.65 (4.24)	9.78 (5.99)	**<0.05**
Gestational age	***<=38 weeks***	***>38 weeks***	***P value***
N (%)	18 (30.5%)	41 (69.5%)	
Mean (SD)	8.13 (5.74)	8.80 (5.92)	>0.05

Table 3.22. $CD4^+$ T-cell proportion by birth weight, gender, and gestational age

(N=59)	**TCD4 (CD3+/CD4+) (%)**
Mean (SD)	46.71 (8.99)

Median [Min; Max]	46.16 [24.36; 71.30]		
(N=59)	**TCD4 (CD3+/CD4+) (%)**		
Birth weight (kg)	***<=3kg***	***>3kg***	***P value***
N (%)	17 (28.8%)	42 (71.2%)	
Mean (SD)	48.83 (8.64)	45.85 (9.19)	>0.05
Gestational age	***<=38 weeks***	***>38 weeks***	***P value***
N (%)	18 (30.5%)	41 (69.5%)	
Mean (SD)	46.82 (10.88)	46.66 (8.30)	>0.05
Gender	***Female***	***Male***	***P value***
Mean (SD)	48.01 (6.57)	45.64 (10.61)	P>0.05

Table 3.23. $CD8^+$ T-cell proportion by birth weight, gestational age, and gender

(N=59)	**TCD8 (CD3+/CD8+) (%)**		
Mean (SD)	22.58 (6.73)		
Median [Min; Max]	22.43 [8.6; 48.96]		
Birth weight (kg)	***<=3kg***	***>3kg***	***P value***
N (%)	17 (28.8%)	42 (71.2%)	
Mean (SD)	20.83 (4.77)	23.29 (7.39)	>0.05
Gestational age	***<=38 weeks***	***>38 weeks***	***P value***
N (%)	18 (30.5%)	41 (69.5%)	
Mean (SD)	24.41 (8.34)	21.78 (5.93)	>0.05
Gender	***Female***	***Male***	***P value***
Mean (SD)	21.13 (5.92)	23.73 (7.29)	>0.05

Table 3.26. Average proportions of NK cell subsets

N=59	**NK CD3-/CD56+ (%)**	**NK $CD56^{dim}$ (%)**	**NK $CD56^{bright}$ (%)**
Mean (SD)	6.4 (4.9)	5.9 (4.8)	0.5 (0.4)

Median [Min; Max]	5.0 [2.1; 24.3]	4.5 [1.8; 23.4]	0.4 [0.01; 1.8]

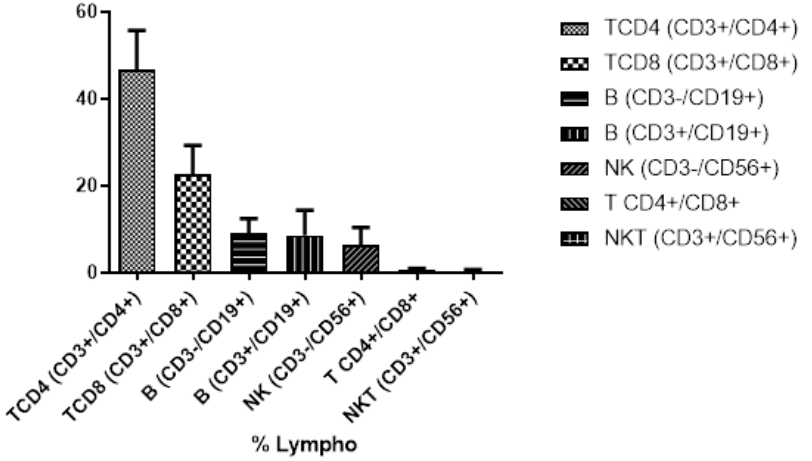


Figure 3.9. Immune cell population proportions in cord blood

3.3.2. The relationship between immune cell subsets and HSC

Table 3.30. Correlation between HSCs and other factors

Factors	**Number of HSC**	
	Correlation coefficient (r)	**P**
Gestational age (weeks)	- 0.08	0.59
Birth weight (kg)	0.14	0.34
Sample volume (ml)	**0.67**	**0.00**
TNCs (10^8)	**0.60**	**0.00**
MNCs (10^8)	**0.57**	**0.00**
CD19+/CD3+ B cells	- 0.16	0.28
B cell (CD19-/CD3+)	**- 0.30**	**0.03**
TCD4 cells	**0.35**	**0.01**
TCD8 cells	- 0.11	0.44
NK cells	-0.23	0.11

Table 3.31. Correlation between factors and the NK proportion

Factors	NK proportion (n=59)	
	Correlation coefficient (r)	P
Gestational age	0.10	0.46
Birth weight (kg)	-0.03	0.83
Sample volume (ml)	- 0.23	0.12
TNCs (10^8)	- 0.01	0.99
MNCs (10^8)	- 0.11	0.46
CD19+/CD3+	- 0.26	0.05
Lympho B (CD19-	0.09	0.50
TCD4 (CD3+/CD4+)	**- 0.42**	**0.01**
T CD8 (CD3+/CD8+)	**- 0.27**	**0.03**

3.4. Activation and expansion of peripheral blood NK cells

Table 3.32 Ex vivo–expanded peripheral blood NK cell product

Cell number at day 21	Vibale (%)	% NK	Number of NK cell (x10^6)	Total cell (x10^6)	N
	(Mean± SD)	(Mean± SD)	(Mean± SD)	(Mean± SD)	
Average	95.0±2.3	88.3±7.8	2,659.3± 1,690.8	3,280.8± 2,444.0	9
Age	*p=0.2962*	*p=0.4275*	*p=0.0734*	*p=0.0759*	
≤ 65	93.7±0.9	85.0±10.7	4,212.0± 2,533.2	5,507.8± 3,865.2	4
> 65	95.3±2.5	89.1±7.3	2,271.1± 1,284.5	2,724.0± 1,787.4	
Cancer type	*p= 0.083*	*p=0.019*	*p=0.256*	*p=0.131*	5
Gastric	95.2±1.1	91.1±9.5	3,255.6± 1,944.3	3,970.8± 2,978.4	

Liver	96.5±0.8	88.2±2.2	1,858.9± 934.2	2,199.8± 1,130.8	3
Lung	91.4±2.7	83.1±11.2	3,067.6± 2,267.2	4,062.6± 3,253.6	3

Table 3.33. Expansion fold

	Expansion fold of total cell	**Expansion fold of NK cell**	**Fold increase in NK proportion**	**N**
	(Mean± SD)	(Mean± SD)	(Mean± SD)	
Average	88.9±38.6	1.132.3±940.3	7.8±6.7	9
Age	*p=0.1962*	*p*=0.0138*	*p*=0.0071*	
≤ 65	115.3±38.0	2.295.2±2.099.3	16.5±11.6	4
> 65	82.3±37.4	601.6±452.5	5.7±2.8	5
Cancer type	*p*= 0.598	*p*=0.000*	*p*=0.014*	
Gastric	110.4±34.8	1.644.9±1510.7	12.3±8.5	3
Liver	69.6±29.0	358.3±94.7	4.3±1.7	3
Lung	84.5±52.7	807.3±695.0	5.8±5.2	3

3.5. Activation and expansion of cord blood NK cells

3.5.1. Selection of the culture medium

he NK cell expansion culture medium manufactured by BINKIT, developed by the Biological Institute of Japan (BIJ), was selected for this study. NK cell products generated using this medium have been previously evaluated for safety and therapeutic efficacy in clinical settings, involving healthy volunteers in Japan and Vietnam. Building upon these prior studies conducted on peripheral blood–derived NK cells, we subsequently employed the same culture medium to expand umbilical cord blood–derived NK cells in the next phase of the study.

3.5.2. Selection of the culture duration

A decline in cell numbers was observed from day 21 of culture onward, similar to that observed in peripheral blood–derived cells; therefore, a maximum culture duration of 21 days was established for all subsequent experiments.

3.5.3. Morphological characteristics of cells during culture

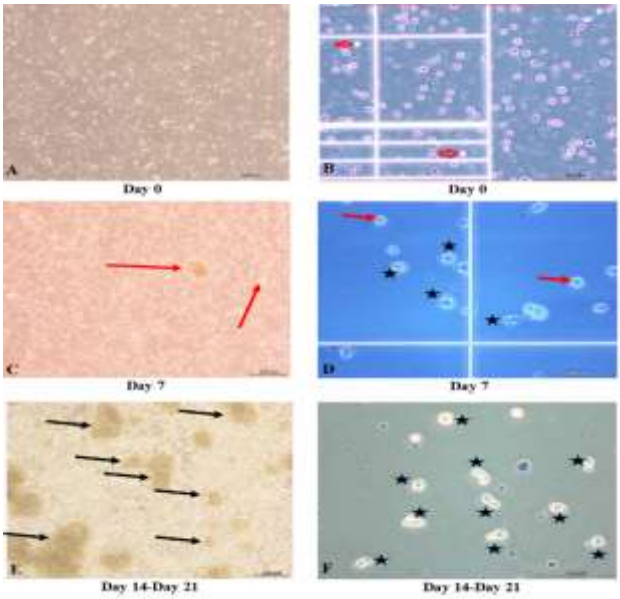


Figure 3.15. Morphological changes of NK cells during culture

From day 5 onward, a reduction in the number of red blood cells in the culture was observed. By days 6–7, the formation of several cell clusters was evident (Figure 3.13C), accompanied by changes in cellular morphology and increased heterogeneity within the cell population. From day 14 onward, both the number and size of cell clusters increased markedly. From day 21 onward, the cells exhibited a tendency toward reduced cell size, concomitant with a decline in overall cell numbers.

3.5.4. Characteristics of the NK cell (CD3-/CD56+) population

3.5.4.1. Immunophenotypic changes of NK cells during the culture process

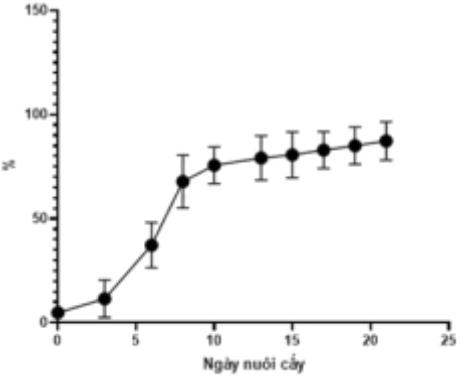


Figure 3.16. NK (CD3-/CD56+) proportion during culture

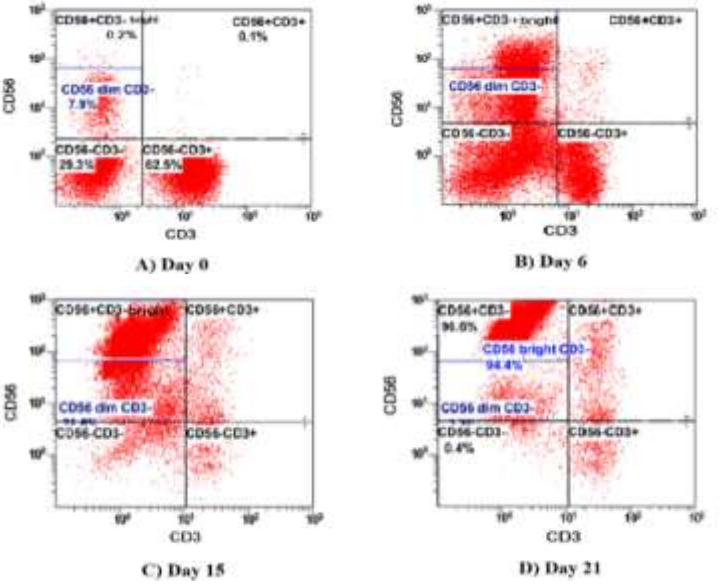


Figure 3.17. Immunophenotype of cord blood NK cells during culture

3.5.4.2. Efficiency of NK cell expansion

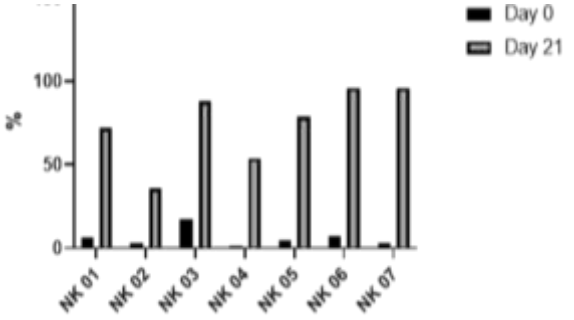


Figure 3.18. Proportion of NK cell before and after culture (n=7)

3.5.5. Immunophenotypes of other cell populations during culture

3.5.5.1. Changes in the proportion HSCs during culture

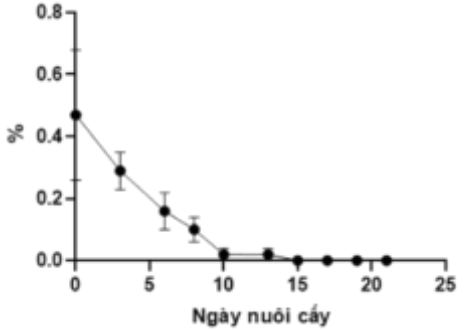


Figure 3.19. Change in HSC proportion during culture (n=3).

3.5.5.2. Selectivity of the NK cell culture medium

Table 3.37. Proportions of immune cell subsets before and after 21 days of culture

	Before	**After**	**N**	**Result**
(CD3-/CD56+)	6.38 (4.87)	77.39 (19.11)	7	**Increase**
(CD3+/CD56+)	0.09 (0.03)	6.21 (7.44)	7	**Increase**
(CD3+/CD4+)	39.51 (11.3)	5.31 (5.03)	7	**Decrease**

(CD3+/CD8+)	20.84 (4.07)	3.47 (3.19)	7	**Decrease**

3.5.6. Changes in cultured cell numbers

Table 3.38. Total cell numbers after 21 days of ex vivo culture

	Total cell	**NK (CD3-/CD56+)**	**N**
Number at D0	45.02 ± 15.75	2.8 ± 1.9	7
Number at D21	2,115.4 ± 1,040.1	1,762.7 ± 1,141.1	7
Increasing fold	46.06 ± 14.21	840.7 ± 550.4	7

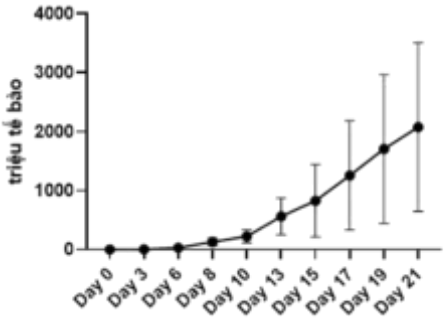


Figure 3.23. Number of cell during culture (N=4)

3.5.7. Doubling time of cell

Table 3.40. NK cell doubling time during different culture phases

Day	**NK's doubling time (days)**				**Mean±SD**	**N**
	Mẫu 1	Mẫu 2	Mẫu 3	Mẫu 4		
Day 3- 10	1.62	1.11	1.14	0.88	1.19±0.31	4
Day 10- 15	4.49	2.22	2.67	3.87	3.31±1.05	4
Day 15- 21	3.53	5.61	3.56	3.60	4.08 ±1.03	4

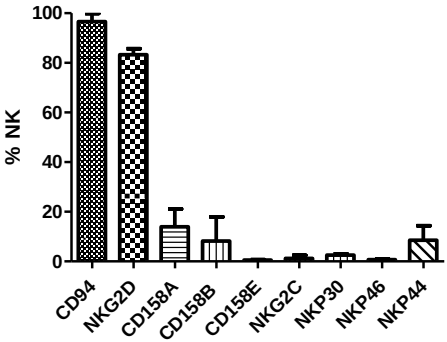
The mean doubling time of NK cells over the entire 21-day culture period was 2.28 days (N = 7), corresponding to 54.82 hours. This value

is higher than that reported for adult peripheral blood–derived NK cells in the study by Yin Liu (2014), which ranged from 1.24 to 1.25 days [21]. It is also higher than the 24–36 hours reported for the commercial NK-92 cell line [22], as well as the 30–40 hours doubling time of the commercial KHYG cell line as reported by the supplier. And this doubling time is substantially shorter than the value reported by Lutz et al. (2011) for NK cells in peripheral blood of healthy adult, which was 13.5 days [23].

3.6. Cytotoxic activity of cord blood–derived NK cells after culture

3.6.1. Expression of activation markers on NK cells

The activating receptor NKG2D was highly expressed, with a mean expression level of 83.2 ± 2.4%, while CD94 showed a similarly high expression level of 96.6 ± 3.3%. In contrast, the inhibitory receptors CD158a and CD158b, as well as the activating receptor NKp44, were expressed at relatively low levels, at 14.0 ± 7.1%, 8.2 ± 9.7%, and 8.5 ± 5.9%, respectively. Meanwhile, CD158e, NKG2C, NKp30, and NKp46 exhibited very low expression levels, each below 3% (Figure 3.25).



Hình 3.25. Expression of activation and inhibition markers on NK cells after culture (n=3)

3.6.2. The ability of NK to kill the K562 cancer cell line

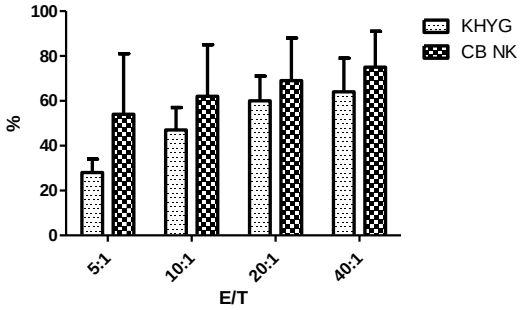


Figure 3.26. NK and KHYG ability to kill K562 cells

With our current culture method, without using foreign cell lines, NK cells after activation and proliferation have a relatively high ability to kill cancer cells, reaching 74.6% (SD=15.7%; N=5) at E/T ratio=40/1, similar to the results of some other studies and NK activity after culture is higher than the commercial cell line KHYG.

3.7. NK viability after thawing

Table 3.42. NK cell viability

Sample	Before storage	After thawing
Sample 01	>90%	87,1%
Sample 02	>90%	46,3%
Sample 03	>90%	81,0%
Average	***>90%***	***71,5% ± 22%***

3.8. Quality of NK cell after activation and proliferation

Table 3.43. Criteria according to FDA standards [169]

Criteria	Requirement	Evaluation

Identification	- The identity of the final biologic product must be verified by assays which should be specific and indicative of the nature or composition of the biological product	- The composition of immune cell types in the product batch was identified using flow cytometry.
Purity and Sterility	- Negative for bacteria and fungi. - Negative for mycoplasma. - Endotoxin content ≤ 5 EU/kg body weight per hour of infusion. - Viability > 70%. - Screening for pathogenic viruses.	- Negative for bacteria and fungi. - Negative for mycoplasma. - Endotoxin content < 0.1 EU/mL. - Post-culture product viability > 90%. - 100% of donors screened for HBV, HCV, HIV, CMV, and Syphilis.
Safety	- The product must be evaluated for safety.	- Cultured cells were assessed for chromosomal stability. - Culture medium composition fully defined and animal-free. - Culture medium safety and efficacy evaluated in phase 1 & 2 clinical trials.
Potency	- The cellular product must be evaluated for functional potential.	- Post-culture cells were assessed for expression of activating/inhibitory markers including CD94, NKG2D, CD158a, CD158b, NKp44, CD158e, NKG2C, NKp30, and NKp46.

		- Post-culture cells were evaluated for cytotoxic activity against the K562 commercial cancer cell line.

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

1. This study analyzed the characteristics and proportions of key immune cell populations in 59 umbilical cord blood (UCB) samples from Vietnamese newborns. Mature B lymphocytes ($CD3^-/CD19^+$) accounted for 9.52% ± 3.28% of total lymphocytes. In infants born before 39 weeks of gestation, the mean B lymphocyte proportion was 8.34% ± 2.36%, which was significantly lower than in infants born after 39 weeks (10.04% ± 3.55%, $p = 0.035 < 0.05$). $CD4^+$ T lymphocytes averaged 46.71% ± 8.99%, $CD8^+$ T lymphocytes averaged 22.58% ± 6.73%, and the $CD4^+/CD8^+$ T cell ratio averaged 0.63% ± 0.41%. Natural killer (NK) cells accounted for a mean of 6.44% ± 4.9%, including two minor subsets: CD56^dim NK cells (5.93% ± 4.81%) and CD56^bright NK cells (0.51% ± 0.39%).

2. The study established an effective protocol for the culture, activation, and expansion of NK cells from UCB. The initial NK cell proportion of 6.38 ± 4.87% increased to 77.39 ± 19.11%, corresponding to a total NK cell count of 1,762.7 × 10^6 ± 1,141.1 × 10^6 after 21 days of culture, representing an 840.7 ± 550.4-fold expansion. Post-culture NK cells demonstrated cytotoxic activity against the K562 leukemia cell line after 3 hours of co-culture: 54.4% ± 26.9% at an E/T ratio of 5:1; 62.2% ± 23.2% at 10:1; 68.6% ± 18.7% at 20:1; and 74.6% ± 15.7% at 40:1. Activation marker NKG2D was highly expressed at 83.2% ± 2.4%, while CD94 expression was 96.6% ± 3.3%. Inhibitory markers CD158a

and CD158b, as well as activating marker NKp44, showed lower expression (14% ± 7.1%, 8.2% ± 9.7%, and 8.5% ± 5.9%, respectively), whereas inhibitory receptor CD158e and activating receptors NKG2C, NKp30, and NKp46 were expressed at very low levels (<3%). Post-culture NK cells after 21 days were free from pathogens, mycoplasma, and had endotoxin levels <0.1 EU/mL; no chromosomal abnormalities were detected. These characteristics preliminarily meet several FDA quality standards for clinical-grade cellular products and provide a foundation for future translational research in humans in Vietnam.

ECOMMENDATIONS

Based on the results obtained, the doctoral candidate also identified several remaining issues that can serve as a basis for future research:

1. Implementing an additional stage for the proliferation and differentiation of hematopoietic stem cells (HSCs) immediately before NK cell expansion and training may further increase the yield of NK cells post-culture.

2. Investigating optimized cryopreservation methods using safe storage solutions free of DMSO and containing biocompatible components may allow direct use of thawed NK cell products without requiring a washing step, which is highly valuable for clinical applications.

3. Further preclinical studies are needed to evaluate the safety and efficacy of the NK cell therapy in animal models prior to human application.

PUBLICATIONS RELATED TO THE DOCTORAL THESIS

1. Chu Thi Thao, Do Thi Hoai Thu, Bui Viet Anh, Truong Linh Huyen, Nguyen Van Phong, Nguyen Thanh Liem and Hoang Thi My Nhung. Efficient Expansion of Human Umbilical Cord Blood- Derived NK Cells Ex Vivo without Requiring Feeder Layers. *Biomedical Journal of Scientific and Technical Research*. 2018, Volume 8 (5): 6739-6743.

2. Chu Thi Thao, Bui Viet Anh, Nguyen Van Phong, Nguyen Trung Kien, Nguyen Dac Tu, Doan Trung Hiep, Nguyen Thanh Liem, Hoang Thi My Nhung. Immune Cells Expansion from Peripheral Blood of Some Vietnamese Cancer Patients. *VNU Journal of Science: Natural Sciences and Technology*. 2021, No 3. Page 1-11

3. Chu Thị Thao, Nguyen Thanh Liem, Bui Viet Anh, Tran Van Hiep, Ly Thi Bich Thuy, Hoang Thi My Nhung, Nguyen Trung Nam. Characterization of cord blood immune and stem cells from Vietnamese infants. 2024, *Vietnam Journal of Biotechnology*, 22(1), 35–44.