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Comparison of Maturation Stages of Natural Killer Cell Differentiation Culture from Cultured and Freshly Isolated Umbilical Cord Blood Hematopoietic Stem Cells

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Abstract

Background: Natural killer (NK) cells originate from the differentiation of hematopoietic stem cells (HSCs) in the common lymphoid progenitor pathway, and HSCs can be obtained from umbilical cord blood (UCB). Comparative studies of NK cell differentiation between cultured and freshly isolated HSCs are important in the development of NK cell therapy for cancer. This study aimed to compare the maturation stages of NK cell differentiation between cultured and newly isolated HSC samples using interleukin-2 in the absence of feeder cells.

Methods: Differentiation cultures were divided into two groups according to HSC source. Giemsa staining and flow cytometry were performed to determine the maturation stages and the presence of NKp46 receptors, respectively.

Results: Giemsa staining revealed that the cultured HSC samples produce a higher number and more mature (stage 5) NK cells than the freshly isolated HSC samples. Flow cytometry showed that the NKp46 mean fluorescence intensity significantly differed between the two samples, and a high level of NKp46 activation receptor was found in the isolated samples on day 35.

Conclusions: The cultured HSC samples could produce more mature NK cell populations than the freshly isolated HSCs, which will be beneficial for the therapy applications of NK cells derived from UCB HSCs.

Keywords: cell differentiation, hematopoietic stem cells, maturation stage, natural killer cells, umbilical cord blood

INTRODUCTION

Stem cells are unspecialized cells that come from the human body and have the ability to self-renewal and differentiate into various types of cells. Hematopoietic stem cells (HSCs) are stem cells that play a role in hematopoiesis in the adult human body. They can be isolated from an adult's red bone marrow and peripheral blood after mobilization byb granulocyte-colony stimulating factor (G-CSF). The ability of HSCs to differentiate into various types of blood cells in abundance can be used to treat multiple types of hematologic cancer.[1] HSCs from umbilical cord blood (UCB) provides the following advantages in clinical applications: (i) low risk of infection due to exposure to an operating theater and prior screening for infections from the mother; (ii) less incidence of graft versus host disease, and (iii) minimal requirement for identical human leukocyte antigen matching.[2]

Natural killer (NK) cells act as antivirals and antitumor as part of innate immunity.[3] They can be detected in varying amounts from primary lymphoid tissues (spinal cord and thymus), secondary lymphoid tissues (mucosa-associated lymphoid tissue, tonsils, and spleen), liver, uterus, and lungs and normally circulate in peripheral blood at a percentage of 5%–15%.[4] NK cells are derived from the differentiation of HSCs in the common lymphoid progenitor pathway, which determines the development of progenitor cells into B or NK/T cells. The presence of CD56 indicates that the final development stage of NK

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cells is the mature (adult) stage, namely, CD56 ^{bright} (stage 4) and stage 5 (CD56^{dim}).[3,5]

NK cell differentiation cultures generally use feeder layers and cytokines. As a feeder layer, the stromal cell line provides microenvironment factors for the maturation of NK cells.[6] Feeder cells increase the number of NK cells for cancer immunotherapy applications but are less beneficial for clinical use for safety reasons, i.e., viral or other intracellular pathogen transmission. Cytokines for NK differentiation culture is the optimal way to obtain a sufficient number of NK cells for cancer immunotherapy applications. Cytokines are necessary for the development, proliferation, and activation of mature NK cells.[7] Dezell *et al*. used various cytokine combinations, including IL-3, IL-7, IL-15, stem cell factor, and Flt-3 ligands, and obtained stage 4 (CD56^{bright}) NK cells on the 35th day.[8] However, their protocol is costly for the further ex vivo expansion of NK cells. This circumstance causes difficulty in the use of UCB HSC-derived NK cells for cancer immunotherapy.

Modification is warranted to develop a simple differentiation protocol capable of producing NK cells at an adult stage; however, comparative studies on the effectiveness of NK cell differentiation with cultured HSCs or freshly isolated HSCs are limited. The differentiation ability and maturation stage of NK cells from HSC cultured samples could be compared with those of NK cells from freshly isolated HSCs to determine the success of the NK cell differentiation protocol.[9] Therefore, this study aimed to compare the maturation stages of NK cell differentiation from HSC cultured or freshly isolated HSCs using a modification of the Dezell protocol. The differentiation protocol in this study used no feeder cells and a single daily cytokine IL-2 supplementation for the culture medium from day 21 to day 35 of differentiation. Gene markers perforin (PRF) and granzyme B (GRB) are generally expressed in some stage 4 NK cells and most stage 5 NK cells. Their expression can be triggered by IL-2 stimulation. The presence of IL-2Rα receptors on activated NK cells can increase the interaction of IL-2 on NK cells.[10] This study used Giemsa staining, flow cytometry, and qRT-PCR to evaluate the maturation stages of differentiated NK cells.

METHODS

Materials

This research was conducted at the Stem Cell and Tissue Engineering (SCTE) Laboratory, Indonesia Medical Education and Research Institute, FK UI, Central Jakarta, from October 2021 to July 2022. This in vitro experimental study compared two treatment groups: HSC samples isolated directly from UCB and HSC samples cultured for 2 weeks and then continued with a culture of differentiation into NK cells for 5 weeks.

HSCs were isolated from two fresh UCB samples (100–120 mL) from patients who underwent cesarean section deliveries at the National Central General Hospital Dr. Cipto Mangunkusumo (RSUPNCM). Sampling was conducted in accordance with the code of ethics (Protocol Number: 21-10-1076) reviewed by the Faculty of Medicine, University of Indonesia. The samples were extracted by a team of ob-gyn doctors at RSUPNCM working with a collaborating researcher, Dr. Gita Pratama Sp. OG(K). For each sample, the number of wells was repeated between 12 and 9 wells on a 12-well plate with two harvesting times for sample analysis on days 14 and 35.

HSC isolation

MNC was isolated from fresh UCB by Ficoll gradient density and centrifugation. The mixture of UCB and Ficoll (ratio volume 1:1) was centrifuged (400 *g,* 10 min, 22°C (*no brake*)). The buffy coat was transferred to a new tube, then washed with PBS with a volume ratio (1:1), and centrifuged (650 *g,* 10 min, 22°C (*low brake*: acc: 5; dcc: 5)). MNC CD34+ antibody was isolated following the immunomagnetic principle of the EasySep kit. Finally, the cells were counted using the Neubauer Improved Counting Chamber.

HSC culture

HSCs were suspended in medium culture (RPMI 1640 + 15% PRP (platelet-rich plasma) + 10% HSA (human serum albumin)) and cultured in each well of a 12-well plate. The method used was derived from optimization in the SCTE copyright laboratory with registration number EC00202201784. The medium was changed every 2 days for up to 2 weeks.

NK cell culture

NK differentiation culture medium was prepared by modification without coculture with immortal liver embryo cell lines and administration of IL-2. The culture medium was prepared using HAM F12:DMEM basal medium with a volume ratio of 1:2 (v/v). The supplements added consisted of 20% (v/v) human serum of blood type A, 50 M ethanolamine, 20 ng/mL ascorbic acid, sodium selenite (Na_2SeO_3), and 100 g/mL pen–strep antibiotics. IL-2 cytokine was added between 21 and 35 days to increase NK cell maturation.[8]

Flow cytometry

Flow cytometry tubes were prepared and labeled "unstained" and "stained" for each NK differentiation culture well and then added with 100 µL of cell suspension. Meanwhile, 3 mM PE-Cy7 conjugated NKp46 was added to the stain tube and resuspended by pipetting up and down. The stain tube was incubated for 15–30 min in the dark. After incubation, 100 µL of PBS was added to each stain and unstained tube. Finally, the sample was analyzed using BD FACS Aria III to obtain the mean fluorescence intensity (MFI). This value is related to the number of antibodies that recognize and attach to cell antigens.[11]

Giemsa staining

Giemsa staining was performed on the 14th and 35th day of harvesting cells to determine the potential of HSCs for differentiation into NK cells. In brief, 200 µL of cell suspension was obtained from each well and dripped on a glass slide, which were then soaked with the methanol-fixative solution and allowed to stand in a staining jar for 30 min. The glass slide was dripped with 5% Giemsa solution and again allowed to stand for 20–30 min. Furthermore, the preparation was rinsed using running water and placed on an upright shelf until it dried. The dried preparations were stored in a preparation box prior to observation using an OPTILAB microscope. Five fields of view were photographed for each slide in each sample repetition to tabulate the number of NK cells present at each stage and compare the stage. The maturation stage of differentiated NK cells was compared with that of NK cells following the method of Freud *et al*.[3]

RNA isolation and cDNA synthesis

RNA was isolated from NK cell culture following the Quick-RNA™ Miniprep Plus Kit reagent protocol [Zymo Research]. NK cells derived from culture and isolation samples were prepared in triplicate. Each sample was derived by incorporating 3–4 wells on 12-well plates for each set of NK differentiation cultures. Purity was measured using a nanodrop to determine the A260/A280 ratio. cDNA synthesis was carried out using the master mix composition in accordance with the TOYOBO manual. The mixture was incubated in a thermal cycler for cDNA synthesis at 37°C for 15 min, at 50°C for 5 min, and at 98°C for 5 min.

The three primers were designed with Primer Blast Tools NCBI, selected and optimized in IMERI. The primers were *actin B*, *granzyme B*, and *perforin* (Table 1). The components of the qRT-PCR mix mixture were entered following the Bioline manual. Amplification began with one cycle of reverse transcription enzyme activation of 45 °C for 10 min, one cycle of polymerase enzyme activation of 95 °C for 2 min repeated 40 times, denaturation at 95 °C for 5 s, and extension of annealing at 56 °C for 10 s and 72 °C for 30 s. CT value on qRT-PCR was processed using the Livak formula.

Data analysis

Data from microscopic observations were displayed in the form of images with a scale to indicate the presence of cells in the culture. Data from Giemsa staining were shown in graphical form to assess the stages of NK cell maturation and tabulate the number of NK cells at each stage. C_T data from qRT-PCR were processed using the Livak formula. Data were analyzed using the normality test, homogeneity test, and t-test to determine differences in the treatment groups using IBM SPSS Statistics 20. Nonnormally distributed and nonhomogeneous data on the expression of perforin, granzyme B, and actin B were analyzed using the nonparametric Mann–Whitney test to determine differences between the two groups.

TABLE 1. Primer sequence

No	Gene	Primer Sequence (5'–3')
1	*ACTB*	F: CATCCGCAAAGACCTGTACG R: CCTGCTTGCTGATCCACATC
2	*PRF*	F: ACAGCTTCAGCACTGACACG R: GATGAAGTGGGTGCCGTAGT
3	*GRZB*	F: CCCTGGGAAAACACTCACAC R: TTACACACAAGAGGGCCTCC

RESULTS

Microscopic observations showed that the HSC structure was round and small (Figure 1a). According to a previous study, HSC morphology is rounder and brighter than its background.[12] NK cell microscopic morphology derived from HSC culture or freshly isolated HSCs showed the morphology of cells that make up colonies (Figures 1b–c). This finding indicated that the HSCs differentiated into other cells. The observed NK cell structure was in the form of a colony due to aggregation in both samples. NK cells have adhesion molecules as a markers of differentiation. One type of such adhesion molecule is very late activation antigen-4 (VLA-4), which triggers the aggregation of NK cells through the LFA-1/ICAM-1 adhesion signaling pathway. VLA-4 expression may increase with the stimulation of recombinant IL-2.[13] The results of Giemsa staining for the cultured and freshly isolated HSC samples showed no difference between the NK cells observed on the 14th and 35th day (Figures 2a–d). Each stage can be distinguished by the size of the cell and the cytoplasmic-to-nucleus ratio of each NK cell. Stage 5 NK cells have a smaller cytoplasmic-to-nucleus ratio than stage 4 NK cells. Granules are prevalent in the cytoplasm of cells at stage 5, although they cannot be observed in each stage 5 NK cell. NK cells in stage 3 are easier to distinguish because they are larger than NK cells in stages 4 and 5.

On the 35th day, stage 5 NK cells were the most abundant in both samples. The number of NK cells in the HSC culture was more than that in the freshly isolated HSC sample (Figure 3). The increase in stage 5 NK cells number on day 35 and the decrease in stages 3 and 4 NK cells on day 35 indicated that NK cells underwent maturation toward stage 5. The stages of maturation of NK cells were in accordance with the research conducted by Grzywacz *et al*., who found NK cells at stages 3, 4, and 5 in CD34+ cord blood samples.[14]

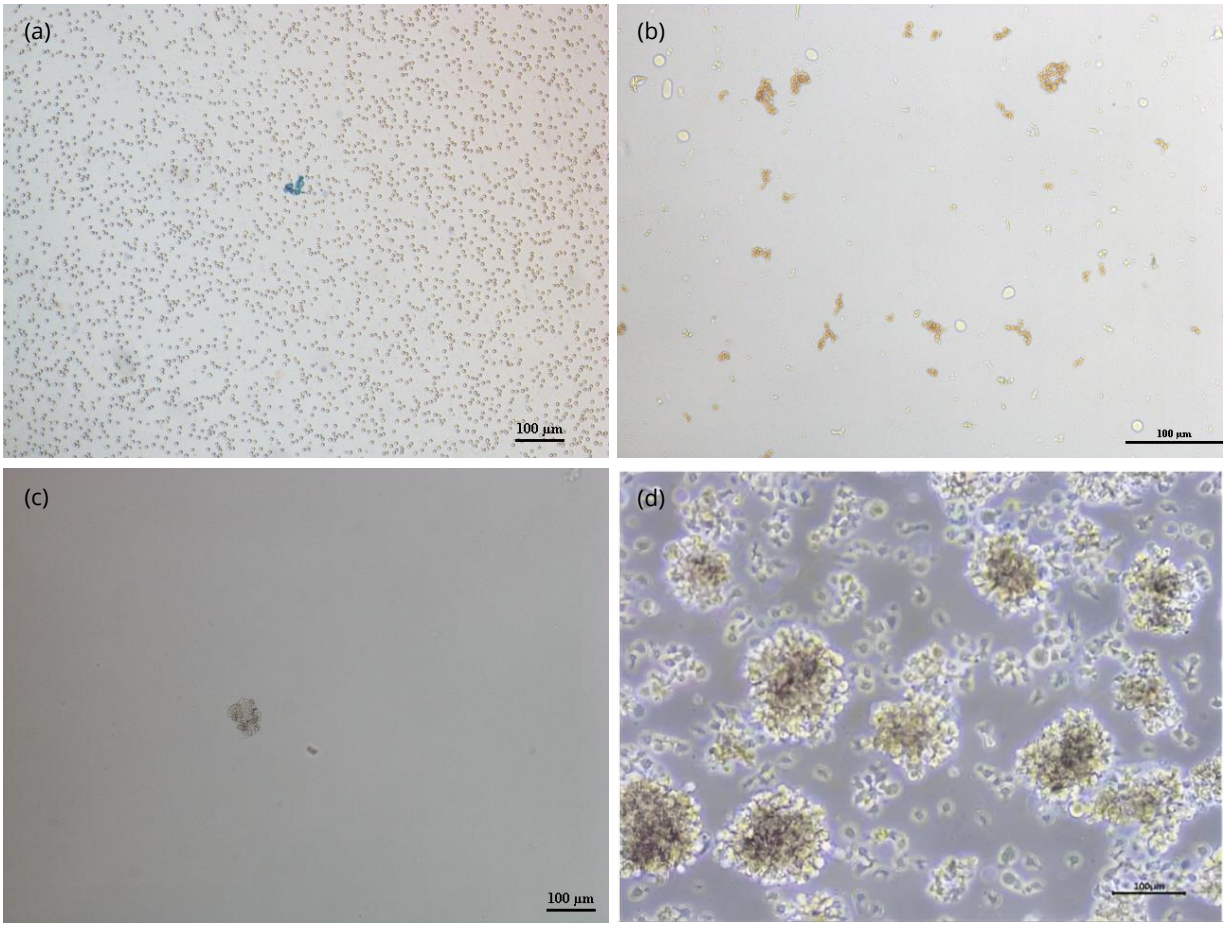
The decrease in stage 3 and 4 NK cells and and the increase in stage 5 NK cells between days 14 and 35 showed that the number of stage 3 ($p > 0.05$; p: 0.70) and 5 ($p > 0.05$; p: 0.97) NK cells was not significantly different

between the two sources. However, the number of stage 4 NK cells significantly differed between the two samples ($p < 0.05$; p: 0.01). Therefore, the number of NK cells commonly found in the HSC culture was influenced by the initial amount of HSCs placed in the well plate (seeding).

Flow cytometry showed the percentage of NKp46 on day 35 of each group sample (Figure 4). The results conformed to the findings of Dezell *et al*.[8] who reported the presence of NKp46 receptors on the 35th day.

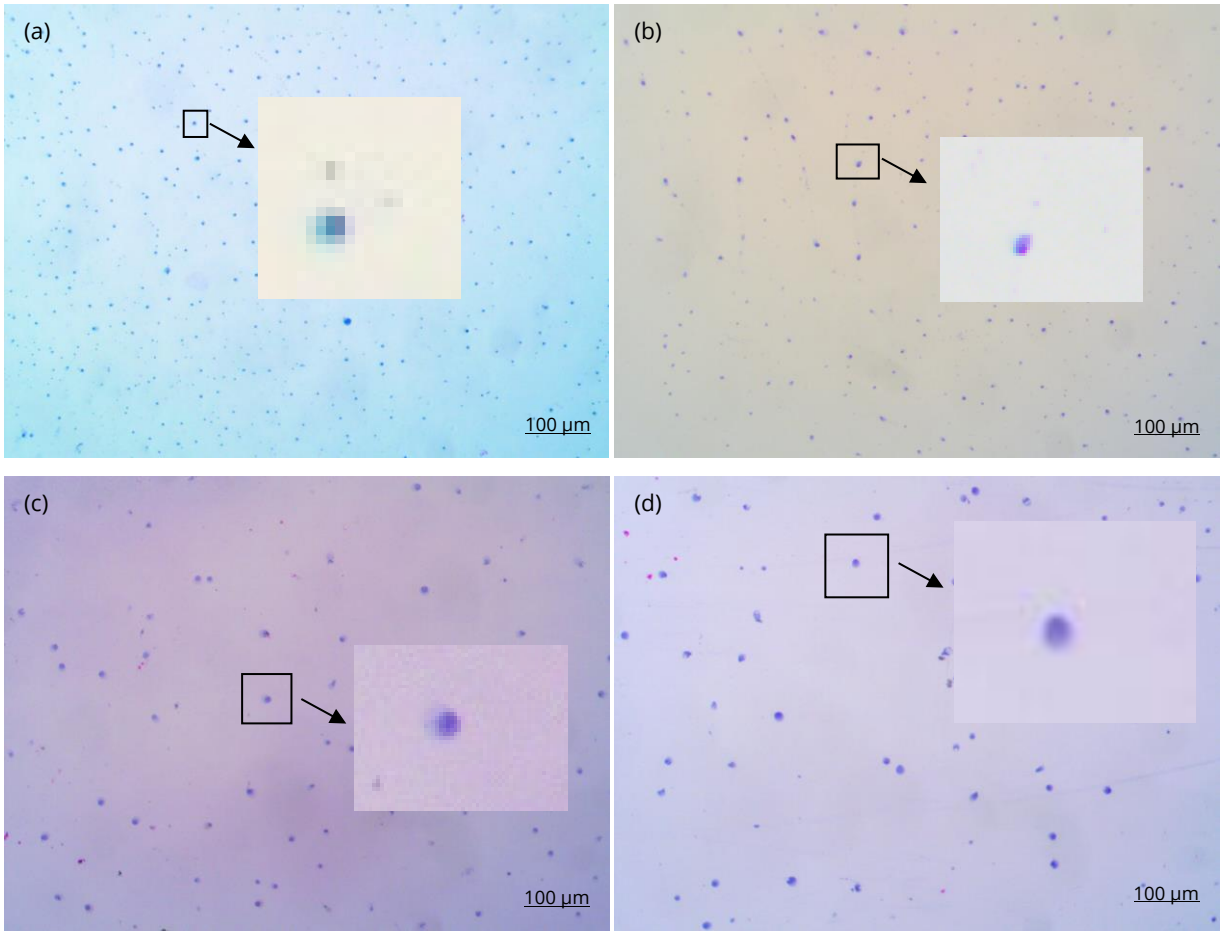
Considering that the percentage of NKp46 reached 100% in both sample groups, the researchers also look into differences in MFI value from each sample (Table 2). Most of the HSC culture samples had MFI in the range of 198–4309, and most of the freshly isolated HSC samples had MFI in the range of 999–7788. Significant difference in MFI ($p < 0.05$; $p = 0.003$) was observed for the NK cells derived from different HSC samples. The high MFI values of the isolated samples indicated that more NKp46 receptors accumulated on day 35 compared with those in the cultured HSC samples. This finding revealed that the differentiation of cultured HSCs in serum-free medium is better than that of freshly isolated HSCs.[9] The administration of IL-2 in vitro can trigger an increase in the expression of NCRs such as NKp46 receptors.[15]

qRT-PCR results showed no significant difference in the PRF expression of NK cells between the two types of HSC samples ($p > 0.05$; p: 0.38); however, significant difference in GRB expression was observed ($p > 0.05$; p: 0.00). This result suggested that PRF and GRB can be expressed in NK cells derived from the differentiation of UCB HSCs.[16] When the samples (differentiation treatment with HSC culture) were compared with positive control (PC), i.e., NK cells, the expression shown was higher. This finding was further reinforced by the greater CD34+ expression (negative controls) showing than NK cells (PC). Therefore, these results cannot be applied to determine the potential differentiation of NK cells from two types of HSC sources.



Magnification 10 x 10, scale bar 100 µm. (a) Morphological Structure of HSC. (b) Results of NK Cell Culture with HSC Isolated Samples. (c) NK Cell Culture Results with HSC Culture Samples. (d) Culture of NK101 in a Culture Medium (Yang *et al.* 2019:5).

FIGURE 1. NK culture microscope observation results for 35 days



Magnification 10 x 10, scale bar 100 µm. (a) Observation of Giemsa on day 14th of HSC Culture samples. (b) Observation of Giemsa on the 35th day of HSC Culture samples. (c) Observation of Giemsa on day 14th of HSC isolation samples. (d) Observation of Giemsa on the 35th day of HSC. isolation samples.

FIGURE 2. Microscopic view by Giemsa staining of culture and isolation samples

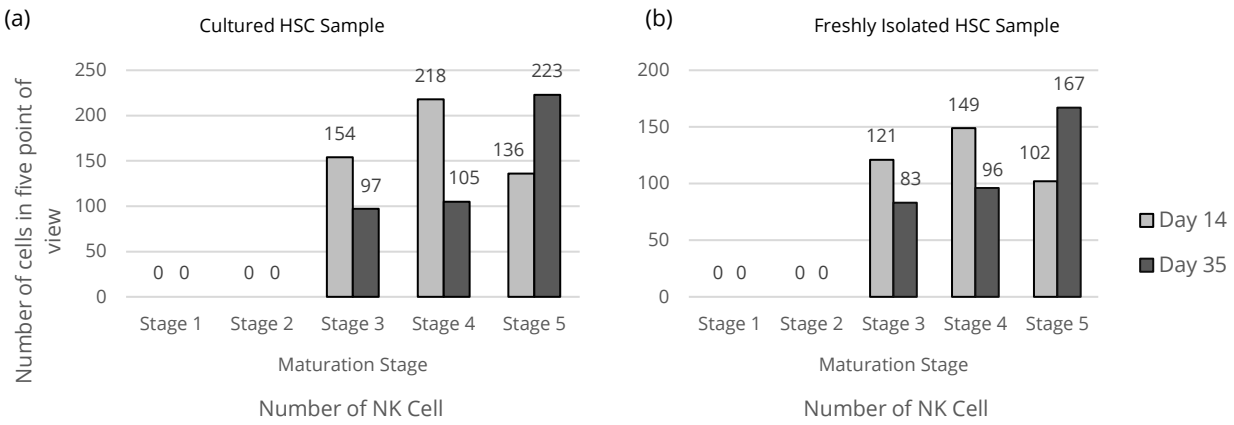
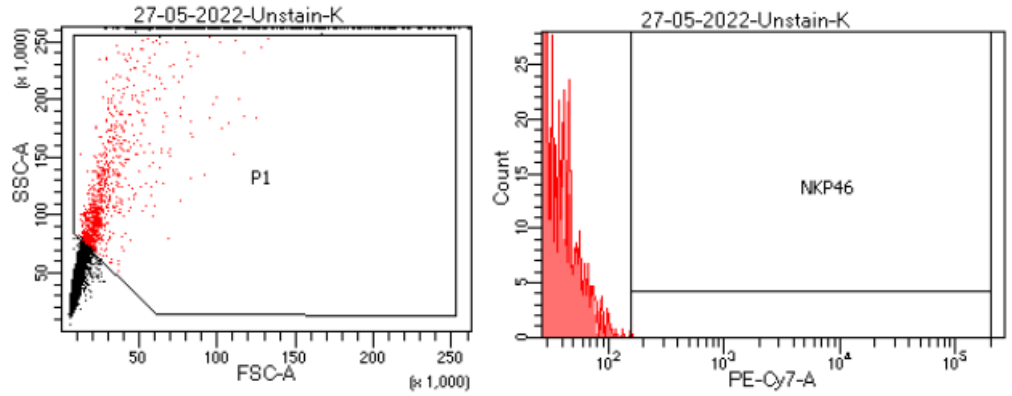
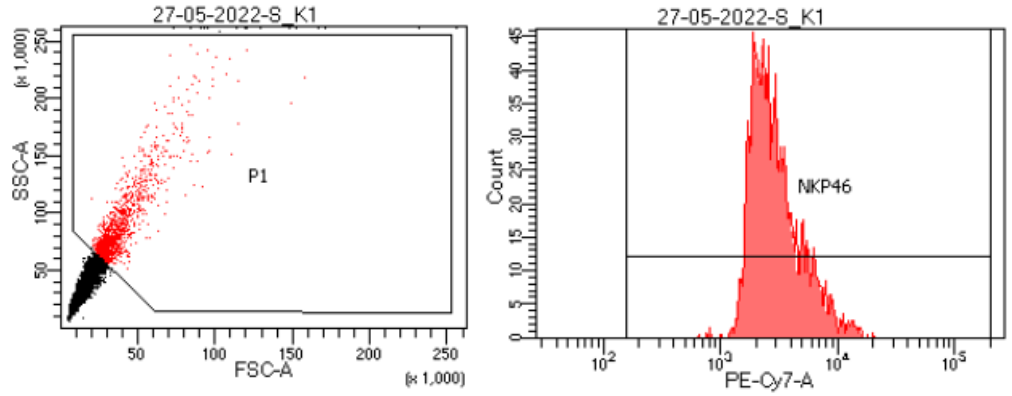


FIGURE 3. Graph of NK cell maturation stages from HSC culture and isolation samples. (a) NK Cell Maturation Stages from HSC Culture Samples. (b) NK Cell Maturation Stage from HSC Isolated Samples.

(a)

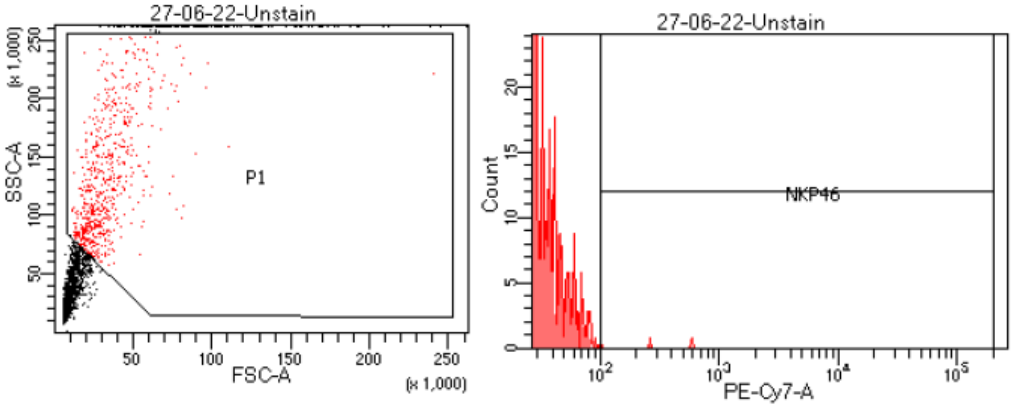


Tube: Unstain-K			
Population	#Events	%Parent	%Total
All Events	5,835	####	100.0
P1	967	16.6	16.6
NKP46	0	0.0	0.0



Tube: S_K1			
Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	1,147	11.5	11.5
NKP46	1,147	100.0	11.5

(b)



Tube: Unstain			
Population	#Events	%Parent	%Total
All Events	2,297	####	100.0
P1	674	29.3	29.3
NKP46	2	0.3	0.1

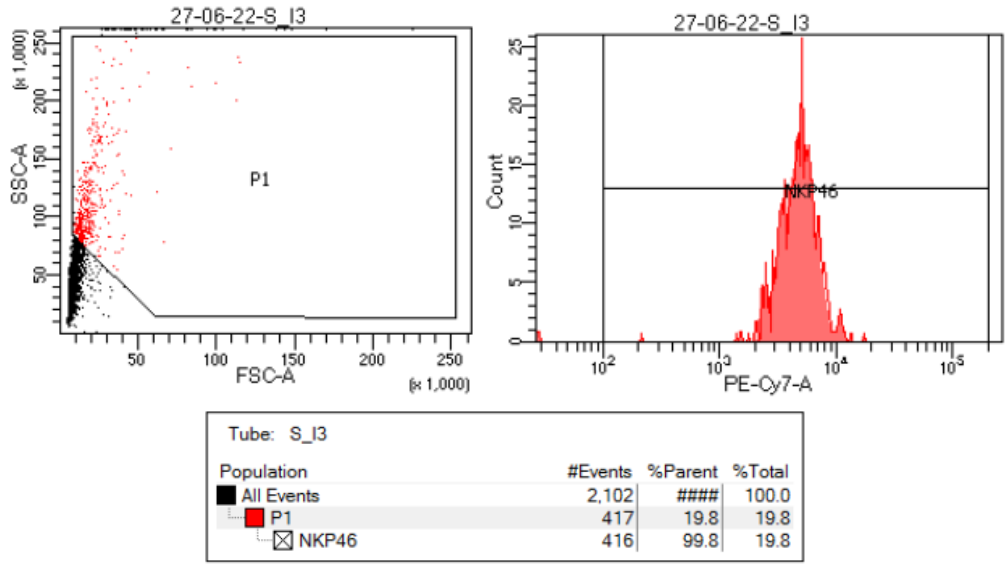


FIGURE 4. Percentage of NKp46 day 35 NK cell culture from (a) HSC culture samples (b) freshly isolated HSC samples

TABLE 2. MFI value differences from day 35 differentiation to day 14 differentiation

Group	Repetition	Differences in MFI value (d35–d14)	Mean	SEM
HSC culture	1	450	1343.25	386.25
	2	2159		
	3	3153		
	4	300		
	5	4309		
	6	852		
	7	507		
	8	2284		
	9	334		
	10	403		
	11	198		
	12	1170		
Freshly isolated HSC	1	4048	3841	639.03
	2	999		
	3	3816		
	4	7788		
	5	4118		
	6	1822		
	7	3439		
	8	3555		
	9	4984		

DISCUSSION

Modifying the existing NK differentiation protocol without using coculture and an immortal liver embryonic cell line with IL-2 can produce stage 5 NK cells on day 35. Dezell *et al.*[8] obtained matured NK cells that were previously in stage 4 after 35 days using stromal cells. In the early weeks, NK cells are predominantly in stage 4 ($CD56^{bright}$) with several types of cytokines, especially without the presence of a feeder layer.[3,17] This finding was supported by the number of stage 4 NK cells at 2 weeks of culture. Maturation can be triggered by the stimulation of IL-2 given in the 3rd week. NK cells that differentiated from UCB HSCs were shown to respond to cytokines, such as IL-2, similar to NK cells in peripheral blood and cord blood.[18]

The determination of the maturation stages of NK cells using Giemsa staining is not necessarily accurate due to subjectivity factors. Determining the stages of development can be performed using other methods, one of which is flow cytometry. One of the markers to decide on the adult stages of NK cells is CD56. $CD56^{bright}$ shows immature adult stage NK cells (stage 4), and $CD56^{dim}$ shows the most mature NK cells (stage 5).[19]

The number of cultured HSCs is greater than that of isolated HSCs. HSC culture serves for cell expansion, so the number of cells produced is high. Meanwhile, the amount of HSC in cord blood becomes limited under isolation. Therefore, the number of NK cells derived from HSC cultures is more significant compared with that from the isolated samples. Calculating the number of NK cells after the culture is terminated is necessary to ensure whether the number of NK cells produced is different according to the HSC-type sample. In general, the number of NK cells is calculated using the trypan blue method adjusted to the percentage of the population of each NK cell using specific receptor markers through flow cytometry.[20]

The low expression of NKp46 can be affected by the presence of reactive oxygen species (ROS).[21] The increase in ROS leads to enhanced proliferation and differentiation activity in HSC.[22] Culturing HSC samples increases the number of cells so that cell proliferation will take place and increase the presence of ROS. An increase in ROS will

trigger toxicity in HSCs, such as DNA damage.[23] The expression of NKp46 receptors can also be affected by microRNA (miRNA) regulation.[24] Cultured HSC samples are known to be hypermethylated, which can alternate the regulation of HSC gene expression and trigger the loss of potential differentiation in most of their progenitor cells. Although NK cells have the same immunophenotypes as other NK cells, they are not necessarily functional.[25] Therefore, further functional tests must be carried out to determine the activity of NK cells from differentiation cultures from both types of HSC samples.

C_T values in some samples could not be determined due to the lack of target genes in the samples[26] influenced by low RNA purity or low RNA concentration. Hence, the obtained data are not representative, except for the negative control that is relatively pure (A260/A280: 2). In addition, the RNA concentration obtained in this study was valued at <5 µg, which is below the recommended range (10–15 µg) for optimal cDNA synthesis. Therefore, the results of qRT-PCR were affected.[27]

On the basis of observations from Giemsa staining, NK cells derived from cultured and freshly isolated UCB HSCs can produce NK cells at stage 5. Therefore, many NK cells can be produced using simple materials (one cytokine and no feeder cells). This synthesis method of NK cells is safer for clinical applications because it reduce some of the possible side effects caused by feeder cell contamination.[9,28] NK cells derived from cord blood are limited and generally immature, making them less beneficial for clinical applications. Some studies have reported successful expansion and functionality of NK cells from UCB HSC with various protocols using the combination of cytokines and feeder cells.[30] In the current work, NK cell differentiation cultures from cultured UCB HSCs can also increase the number of NK cells required for clinical applications.

CONCLUSIONS

Observations from Giemsa staining showed that NK cell differentiation culture without feeder cells and using IL-2 produced more stage 5 NK cells after 35 days for HSC culture samples than for freshly isolated HSC samples. The obtained NK cells also have the NKp46 receptor on day 35. These results show that HSC culture can enrich mature NK cells produced in vitro, which can later be used for immunotherapy applications. In addition, HSC culture can enrich the number of mature NK cells, which can later be used for immunotherapy applications. Functional test on the ability of NK cells is warranted for their future clinical applications.

CONFLICT OF INTEREST

The authors declare that no conflicts of interest arise in the scientific dissemination of this paper.

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